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Effects of Heavy Metals on the Differentiation of Metabolic Pathways in the CNS

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Philadelphia, PA

Prepared for

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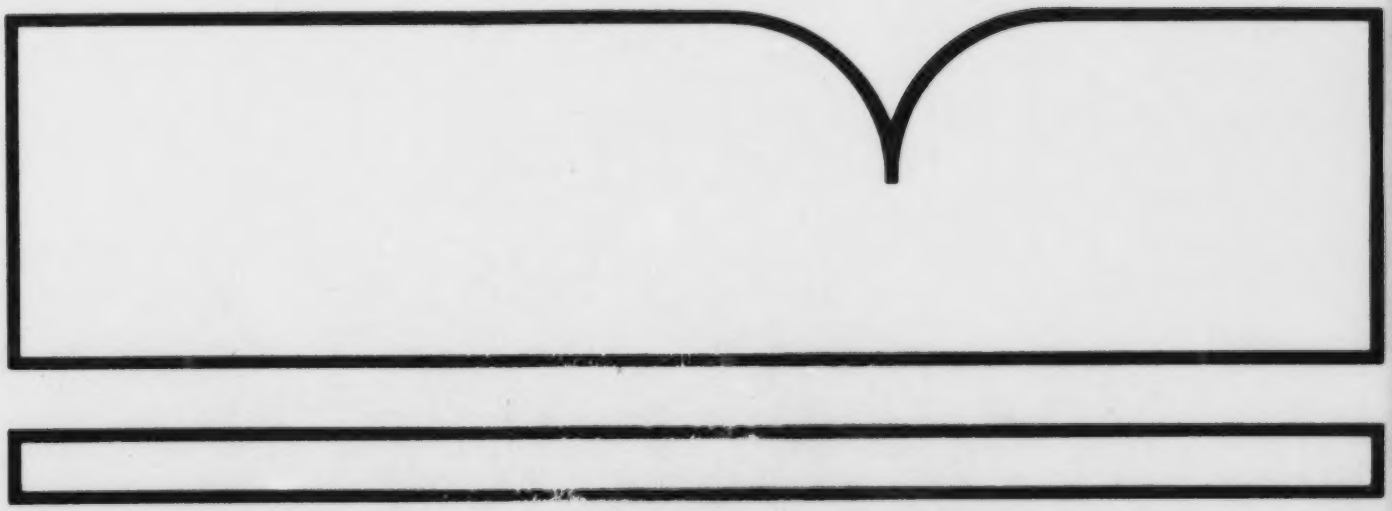

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16. ABSTRACT

The effects of lead and organotin on brain and spinal cord, liver or kidney in the developing or adult Sprague-Dawley and Wistar rat strains were investigated. Two concentrations of lead were present in drinking water: 200 parts per million or 600 parts per million as lead in lead acetate. Blood and tissue lead levels were also measured.

Tissue slices prepared from kidney cortex of animals exposed to 200 or 600 ppm lead showed a respiration rate 15% less and ATP contents which were 30% less when compared with corresponding tissue from control animals. Lead treatment *in vivo* was found to have no significant effect on the ion and water contents of kidney cortex in contrast with results obtained when lead was added *in vitro*. The ability of kidney cortex slices to restore ion and water contents after incubation at 1°C was also unimpaired after *in vivo* treatment.

Marked differences have been shown *in vitro* with respect to the lead sensitivity of carbonic anhydrase, a zinc-enzyme which appears organ specific. At a lead concentration of 10 parts per million, there is a 50% fall in myelin carbonic anhydrase and a 44% fall in kidney carbonic anhydrase. The enzyme present in nerve endings is particularly sensitive and shows a drop from 20 units ± 4 to 2.3 ± 1.7 units. The nerve ending enzyme is sensitive even at 1 ppm. With *in vivo* administration of lead (600 ppm) myelin and synaptosomal activity were decreased 67% and 57%, respectively, whereas kidney microsomal carbonic anhydrase showed a relatively small change (19%).

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EFFECTS OF HEAVY METALS ON THE DIFFERENTIATION OF METABOLIC PATHWAYS IN THE CNS

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The complexities of the environmental problems originate in the deep interdependent relationships between the various physical and biological segments of man's natural and social world. Solutions to these environmental problems require an integrated program of research and development using input from a number of disciplines. The Health Effects Research Laboratory conducts a coordinated environmental health research program in inhalation toxicology, genetic toxicology, neurotoxicology, developmental and experimental biology and clinical studies using human volunteer subjects. These studies address problems in air pollution, water pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides and other chemical pollutants. The Laboratory participates in and provides data for the development and revision of criteria documents on pollutants for which national ambient air quality and water quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of environmental regulatory decisions involving the protection of the health and welfare of all U. S. inhabitants.

Lead is a long established pollutant of the environment and as a result has been studied more thoroughly than any other chemical. In the past decade attention in lead toxicology has refocused on the developing animal as a result of epidemiological studies that suggest a high sensitivity of the developing nervous system to lead. This report represents part of the effort expended by the Health Effects Research Laboratory in Cincinnati to develop a better data base for establishing acceptable levels of lead in drinking water.

Tin compounds were the other major concern in this work. A number of alkyl tin compounds, usually the methylated and butylated derivatives, are utilized as stabilizers in PVC pipe used as potable water service lines. The toxicity of these compounds has been poorly characterized, but a related compound, triethyltin is known as a very potent central nervous system toxin. Consequently, the results of some initial work in this area is reported as well.

F. G. Hueter, Ph.D.
Director
Health Effects Research Laboratory

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ABSTRACT

The effects of lead and organotin on brain and spinal cord, liver or kidney in the developing or adult Sprague-Dawley and Wistar rat strains were investigated. The effects were primarily ascertained in vitro following lead exposure. Two concentrations of lead were present in drinking water: 200 parts per million or 600 parts per million as lead in lead acetate. Blood and tissue lead levels were either measured by anodic stripping voltammetry or by atomic absorption spectrophotometry (Perkin Elmer Model 303) following digestion in nitric acid.

Tissue slices prepared from kidney cortex of animals exposed to 200 or 600 ppm lead showed a respiration rate 15% less and ATP contents which were 30% less when compared with corresponding tissue from control animals. Lead treatment in vivo was found to have no significant effect on the ion and water contents of kidney cortex in contrast with results obtained when lead was added in vitro. The ability of kidney cortex slices to restore ion and water contents after incubation at 1°C was also unimpaired after in vivo treatment. It is possible that longer in vivo exposure might be necessary for effects to become evident.

Marked differences have been shown in vitro with respect to the lead sensitivity of carbonic anhydrase, a zinc-enzyme which appears organ specific. At a lead concentration of 10 parts per million, there is a 50% fall (5.11 ± 1.46 units to 2.54 ± 0.75 units) in myelin carbonic anhydrase and a 44% fall in kidney carbonic anhydrase. The enzyme present in nerve endings is particularly sensitive and shows a drop from 20 units ± 4 to 2.3 ± 1.7 units (i.e., to 12% of normal). The nerve ending enzyme is sensitive even at 1 ppm. In vivo administration of lead (600 ppm) was carried out over a 3 month period and brain myelin, synaptosomal and kidney microsomal carbonic anhydrases were measured. Consistent with in vitro measurements, myelin and synaptosomal activity were decreased 67% and 57%, respectively, whereas kidney microsomal carbonic anhydrase showed a relatively small change (19%) from 9.46 units to 7.6 units following 12 weeks exposure.

This report was submitted in fulfillment of Contract No. 68-03-2381 by Temple University School of Medicine, Department of Pharmacology, under the sponsorship of the U.S. Environmental Protection Agency. This report covers the period April 1, 1976 to March 31, 1979 and work was completed as of March 31, 1979.

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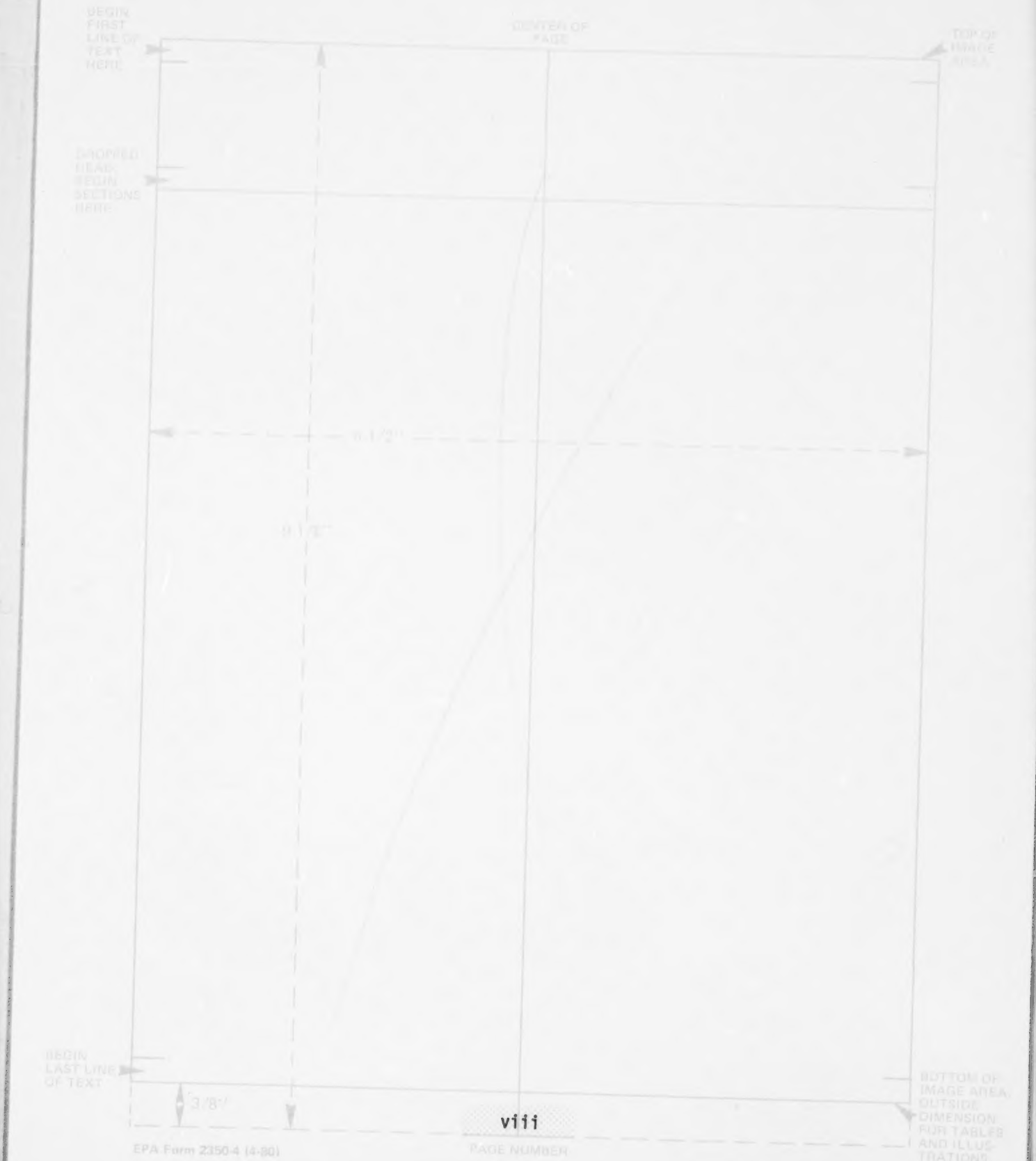

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SECTION I

INTRODUCTION

The neurotoxic effects of lead, mercury, and organotin are well established at high dose levels. The controversy and source of concern is not with high concentrations but rather with levels that do not cause overt symptoms. This appears to be especially true in the case of lead intoxication where the symptoms are not well established. The effects on adults are qualitatively different from those on young children and the developing nervous system (Needleman et al., 1979).

The work described in this final report describes progress made during the contract period concerning the central and peripheral effects of controlled exposure of pregnant and nonpregnant albino rats to levels of 200 or 600 parts per million of lead and tributyl-tin.

The research was divided into three group projects. The principal investigator concentrated his laboratory's efforts on the influence of lead on the developing and adult central nervous system. Considerable emphasis was devoted to monitoring the morphology by electron microscopy and the neurochemistry of the developing spinal cord. Although the brain and especially the cerebellum appear to be the main targets of lead, the spinal cord undergoes development earlier, and it was thought that lead effects in pregnancy might first be manifest there.

A perusal of the current literature suggested that lead and organotin compounds might affect two aspects of metabolism and account in part for their toxic effects (Goyer and Krall, 1969; Aldridge and Cremer, 1955). These effects are (1) energy production of cellular activities, and (2) the regulation of cellular ionic composition and tissue volume (i.e. edema). Since nephrotoxicity is a prominent manifestation of lead poisoning, the use of kidney as well as hepatic tissue in these studies are especially relevant.

The role of the ubiquitous enzyme carbonic anhydrase is poorly understood. In kidney tissue, it appears to assist in maintaining H^+ ion and electrolyte balance. Although present in the intra-lamina of myelin in all nerve tissue and thought by some investigators to be involved in controlling cerebral and spinal tissue edema, there is no general agreement in this regard. Because lead and some organotin compounds, especially triethyl tin, are known to cause cerebral edema, some attention was devoted to various forms of the enzyme which occurs in brain and kidney.

Tissue lead has routinely been measured by flame absorption spectropho-

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tometry. This may require the use of the major portion of a sample by standard instrumentation or miniaturization of the system which requires a considerably more expensive investment in apparatus. In search of alternative methods we elected to study blood, brain, kidney, and liver tissue lead levels by Anodic Stripping Voltammetry. The basic unit is relatively inexpensive, is portable, and requires relatively small (100 microliter) sample size. The method is highly versatile and can be employed in measuring such metal ions as lead, tin, cadmium, iron, or its chelates.

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SECTION 2

CONCLUSIONS

The contract has allowed our laboratory to initiate a study of the effects of lead and organotin compounds which perusal of the literature had suggested might be responsible for some of the toxic effects of these materials. These include the provision of energy for cellular activities, and the regulation of cellular composition and volume. Our results show that lead inhibits energy metabolism in kidney cortex and specifically alters K^+-ion transport and Ca^{+2}-ion distribution in the cells, and we suggest that these factors may play a role in the nephrotoxic manifestations of lead poisoning.

In contrast to findings of others in synaptic development in the cerebral cortex and the toxic effects of lead in vivo, no significant differences were found in synaptic development in the thoracolumbar region of the developing spinal cord. Inability to demonstrate a gross morphologic effect may be due to the lack of specificity of the method. Although some features of the pre- and postsynaptic apparatus can be discerned, the method does not permit a study of the various receptors known to be present. Employing the potent muscarinic binding drug, Quinuclidinyl benzilate-^{3}H (QNB-3), which has high affinity for muscarinic receptors ($K_{aff}=10^{-13}M$) lead (600 ppm), in drinking water consumed by the pregnant and/or nursing dam caused a slight delay in the appearance of muscarinic binding sites in spinal cord over the course of fetal and early neonatal development (Fig. 12,13).

Lead poisoning can produce a devastating encephalopathy in children characterized by increased intracranial pressure (ICP), convulsion and coma. The metabolic basis is not understood but the disruption of fluid and electrolyte metabolism undoubtedly is involved in the development of edema responsible for the ICP. It is interesting to note in passing that in studies on liver employing tributyl-tin that though this compound has a weakly inhibitory effect on energy metabolism, it possesses a hitherto unknown effect as an inhibitor of cell volume regulation in liver cells. We suggest that this latter effect may underlie the edematous changes observed by previous workers in the central nervous system of animals poisoned with trialkyl-tin compounds and possibly by lead as well.

Experiments in which lead (600 ppm) was administered to rats in drinking water for several weeks showed that treatment in vivo reduced the respiratory activity (by 15%) and ability to maintain ATP levels (by 30%) of intact cells of kidney cortex when incubated as a slice preparation (Fig. 2). This is consistent with findings by others on mitochondria isolated from such

kidneys and our results show their findings to be relevant to intact cells. Further, when additional lead was added *in vitro* to the slices, an extra fall in respiration and ATP levels was produced. The minimal levels thus obtained however, were not different from those found by direct treatment of slices from control rats with lead *in vitro* (Fig. 2). This finding indicates that lead *in vivo* and *in vitro* were not additive in effects and were probably acting at the same site. In view of the rapidity of action *in vitro*, we believe lead acted directly as an inhibitor or uncoupler of mitochondrial oxidative phosphorylation. Similar findings in brain slices from rats treated for 20 days at 200 and 600 ppm lead also occur, and help to explain effects on acetylcholine synthesis (see below).

The effects on oxidative phosphorylation were observed in studies on mitochondria isolated from kidney cortices of control animals, in which lead added only *in vitro* had marked inhibitory effects on oxidative metabolism (Fig. 5b). Similar results on rat brain mitochondria from untreated animals were obtained showing brain mitochondria to be exquisitively sensitive to lead ions. These results in adult animals thus all argue against the possibility that lead poisoning *in vivo*, at least for the period of our study, may reduce the synthesis of cytochromes (by way of its known effect on brain synthesizing enzymes) sufficiently to produce a marked effect on cell respiration. Rather, we believe that lead had a direct effect on respiratory metabolism, the precise nature of which is being further investigated.

The administration of lead *in vivo* affected neither the water and ionic (K^+, Na^+, Cl^-, Ca^{2+}, Mg^{2+}) composition of the kidney cortex *in vivo*, nor the ability of slices of the tissue to transport any of these ions or water. Thus, the reduction of energy metabolism by the lead treatment *in vivo* was insufficient to limit the supply of ATP required for these homoeostatic mechanisms and, further, the levels of lead attained in the tissue were insufficient to cause any direct inhibition of the transport mechanisms.

Currently, rats are being treated with lead for longer periods (up to six months) to see whether any extra effects are produced.

The addition of lead to kidney-cortex slices *in vitro* at 50-200 μM, had two effects on ion exchanges: First, lead *in vitro* reduced, by a maximum of 30% the active accumulation of K^+; there was no significant effect on the net extrusion of Na^+, Cl^-, Ca^{2+} or water, or on the tissue content of Mg^{2+} (Tables 1 and 2). The partial inhibition of K^+ accumulation appeared rather late during incubation, becoming apparent only after 30 min (Fig. 1b).

The concentration of lead (200 μM) causing the 30% inhibition of K^+ accumulation also gave 25-30% inhibition of respiration and fall of ATP content, and the time-course of its effect on ATP closely followed the effect on K^+ transport (Fig. 1b). Thus, there is circumstantial evidence that the effects on energy metabolism, by reducing the availability of ATP, were responsible for the transport inhibition. However, the lack of effect on Na^+ extrusion was rather unusual for such a mode of action of inhibitor. Moreover, lead is known to be an inhibitor of the Na^+ and K^+-dependent adenosine triphosphatase (Na-K-ATPase) of kidney and other cells, and this effect could have accounted directly for the transport inhibition. To distinguish be-

tween these two possibilities, kidney cortex slices were titrated with a specific inhibitor of mitochondrial respiration, antimycin A, and with an inhibitor of the Na-K-ATPase, ouabain, so as to permit us to compare the effects of these agents when K^+ transport was inhibited by 30% with the effects of Pb^{2+} giving the same reduction of K^+ transport. However, the comparison gave equivocal results. When antimycin A was the inhibitor, 30% inhibition of K^+ transport was accompanied by a similar fall of respiration and ATP contents but, unlike the situation with lead, Na^+ extrusion was also inhibited(Fig.3a,b). When ouabain gave 30% inhibition of K^+ transport, the extrusion of Na^+ was not inhibited while respiration fell by 25% (Fig. 4), results similar to those with Pb^{2+}; ATP was unchanged by ouabain. The biggest difficulty in explaining the results with Pb^{2+} on the basis of this comparison lies in the failure of a 30% reduction of ATP levels produced by lead to cause any inhibition of Na^+ extrusion. The temporal coincidence of the effects of lead on K^+ transport and ATP levels (Fig.1b & 2b) suggest that the latter accounts for the former, but it is clear that further experiments are required to elucidate the mechanism by which Pb^{2+} inhibits K^+ accumulation.

The second effect of lead in vitro on ion exchanges was to reduce the entry of Ca^{2+} into the slices under conditions in which metabolic activity was greatly reduced, i.e., at 1°C. Study of the Ca^{2+} content of mitochondria isolated from the slices showed that a substantial proportion of the effect of lead was to prevent the entry of Ca^{2+} into these organelles. This was apparently due to inhibition by lead of a residual, calcium-accumulating activity of the mitochondria which persisted at 1°C (Table 3). However, this did not account for the whole of the reduction by lead of the entry of Ca^{2+} into the whole slice at this temperature. Studies of ^{45}Ca influx and efflux in the slices were, therefore, performed in order to obtain more information on possible fractions of tissue Ca^{2+} that might be affected by lead. We found that lead reduced the quantity of Ca^{2+} which was inexchangeable with ^{45}Ca in the medium, and also reduced the quantity of Ca^{2+} in the most slowly exchanging component of the three into which the exchangeable Ca^{2+} could be divided (Fig. 7; Table 4). We suggest that the latter probably represents the mitochondrial Ca^{2+}, so that this result complements our findings with the directly measured Ca^{2+} on mitochondria. The inexchangeable portion may represent Ca^{2+} which is so tightly bound to various nonmitochondrial tissue structures that it cannot be displaced by ^{45}Ca, but which may be displaceable by Pb^{2+}. This latter fraction would then account for the nonmitochondrial portion of the Ca^{2+} uptake that was prevented by lead in the experiments of Table 3.

Initial studies in our laboratory investigated the pathway of metabolism leading from glucose to acetylcholine synthesis. It is well established that brain and nervous tissue cannot synthesize choline and must depend on an exogenous source for supply. Equally well established is the fact that glycolysis supplies pyruvate as a precursor for Acetyl-CoA and ultimately acetylcholine formation. The problem which has confronted neurochemists for two decades is that pyruvate forms Acetyl-CoA intramitochondrially and as such it remains within the mitochondrion. This is because no known mechanism exists for its transport to the cytoplasm, the site of acetylcholine synthesis.

We recently demonstrated that citric acid formed intramitochondrially leaves and enters the cytosol. There it is cleaved by an enzyme ATP: citrate lyase to reform Acetyl-CoA and thence to form acetylcholine (G.H. Sterling, Ph.D. Thesis, OSU 1977; G.H. Sterling and J.J. O'Neill, J. Neurochem., 1978). We have determined that lead at either 200 ppm or 600 ppm in drinking water results in the inhibition of acetylcholine synthesis. We find that at 600 ppm there is a marked decrease in the conversion of glucose to lactate and citrate. Hence, lead interference with glycolysis and the tri-carboxyllic cycle (TCA) may interfere with energy metabolism and affect neurotransmitter function.

The mechanism by which acetylcholine is derived from glucose and pyruvate has been intimately linked to brain mitochondria since the enzyme which converts pyruvate to acetyl-CoA (Pyruvate dehydrogenase E.C. 1.2.4.1) exists exclusively intramitochondrially. Acetyl-CoA thus formed must be converted to a permeate anion in order to cross the mitochondrial membrane to the cytosol (Fig. 9). The evidence to date strongly supports citrate as that anion; when citrate transport is interfered with (J. O'Neill and G. Sterling, 1978) acetoacetate fulfills this role. The approach used to test this hypothesis is based on the observation that glucose labeled in the carbon-6 position with either tritium (^{3}H) or carbon-14(^{14}C) is converted to pyruvate without loss of either isotope. However, when acetyl-CoA is condensed with oxaloacetate (OAA) (Fig. 10), a hydrogen is lost to OAA which is exchanged and lost to water in the formation of citrate. The probability that the hydrogen lost would be tritium, barring isotope discrimination, would be one-third and that would reduce the $^{3}H/^{14}C$ ratio to 2/3 (0.67) (Fig. 11). This ratio remains constant only in citrate carbons 4 and 5, and is drastically reduced in other molecules with each turn of the cycle within mitochondria. Acetyl-CoA arising from citrate (C-4,5) in the cytosol gives rise to acetylcholine with the same $^{3}H/^{14}C$ ratio (0.67); direct conversion from glucose through acetate the ratio would remain unity: (Fig. 8).

Tributyl-tin was studied for its effects in vitro on rat liver, since most biochemical studies of the organotin compounds have concentrated on the mitochondria isolated from this tissue. In spite of the reported high sensitivity of several aspects of oxidative metabolism in isolated mitochondria to tributyl-tin, we needed a concentration of 1 mM to produce a 20% fall of respiration and ATP content in slices of liver in vitro (Fig. 15). At this concentration, there was a substantial decline in the net transport of K^+, Na^+, Cl^- and water.

Much lower concentrations of tributyl-tin (1-100 µM), however, significantly reduced the extrusion of Cl^- from the liver slices, an effect accompanied by rather less marked inhibitions of Na^+ and water extrusion (Fig.16). But K^+ accumulation was completely resistant to these concentrations of the tin compound. We conclude that tributyl-tin at 1-100 µM was probably acting as a rather specific inhibitor of the mechanism for net extrusion of Na^+ and Cl^- which is not coupled to K^+ uptake and which is partly responsible for the control of cell volume. This effect may be related to the known effect of tributyl-tin in facilitating Cl^-/OH exchanges through biological membranes. Higher concentrations (1 mM) also affect mitochondrial energy provision, and thus inhibit the ouabain-sensitive transport of Na^+ and K^+ by the paucity of

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ATP. If tributyl-tin is indeed a specific inhibitor of the Na^+ and Cl^- transport not coupled to K^+, it may well prove to be a most useful agent with which to study the properties of this system, and hence, to study volume control. Its action as an inhibitor of volume control may well account for the finding of manifestations of edema in the central nervous system of animals intoxicated with trialkyl-tin compounds.

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SECTION 3

RECOMMENDATIONS

The studies on lead have extended knowledge of the mechanism of action of this heavy metal and may be particularly relevant to the means by which it causes nephrotoxicity. We have indicated that lead acts on energy provision *in vitro* by a mechanism similar to that by which it acts *in vivo*. Lead causes alterations of K^+ homeostasis in the kidney cells and also affects the distribution of Ca^{2+} in them, especially with regard to the compartmentalization between mitochondria and other cell components. Such effects on ion homeostasis, especially that of Ca^{2+}, may well lead to far-reaching alterations of metabolic balance and of reabsorptive and excretory activities of the kidney cortex.

In light of the findings on brain metabolism by us and others and recent clinical evidence that there is a strong correlation between lead exposure and minimal brain damage characterized by hyperactivity that may interfere with classroom performance (New England Journ. of Med., 300:689-695, 1979) further studies on this important aspect is warranted. The social implications of present drug treatment of hyperactivity should be reappraised and greater emphasis be placed on monitoring of safe lead levels in drinking water to prevent such neurophysiologic damage in future young.

The action of tributyl-tin as an inhibitor of volume-controlling activity, at concentrations substantially lower than those at which it affects energy metabolism in the intact cells, provides a completely new indication of its possible mode of action. Such an action, if it also occurs in nervous tissue, could readily provide an explanation of the central nervous toxicity of the trialkyl-tin compounds. This line of investigation may well prove to be more profitable in elucidating the mechanism of toxicity of the trialkyl-tin compounds than studies of energy metabolism and other functions of isolated mitochondria.

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SECTION 4

METHODS

ANIMALS

Male Albino rats of a Wistar or Sprague Dawley strain were used. Animals were either fed ad libitum on a standard laboratory diet or in the case of the pregnancy studies, a special diet prepared according to the special formula devised by Michelson (personal communication) was employed. Those animals used for study of the effects of agents in vitro only were given tap water to drink. For experiments on the effects of lead administration in vivo, groups of 4 control and 4 experimental ("treated") rats were given deionized water containing either sodium acetate (3 mM) or lead acetate (3 mM, equivalent to approximately 600 ppm lead), adjusted to pH 5.5-6.5, to drink throughout the treatment period. For experiments, animals were killed by decapitation and their kidneys and/or liver rapidly removed and immersed in ice-cold Ringer solution (see below).

PREPARATION AND INCUBATION OF TISSUE SLICES (KIDNEY AND LIVER)

(a) Kidney cortex - Kidneys were cut into two through the hilum and the capsule removed from each half. The red medulla was carefully removed with fine scissors and the cortex was sliced with a razor blade guided with a glass slide. Slices were less than 0.4 mm thick and were shown by histological study to contain very little material from the medulla (van Rossum & Ernst, 1978). The slices from the two kidneys of a single animal were randomized and then divided as equally as possible between vessels containing medium (20 ml per vessel) with appropriate concentrations of Pb^{2+} at a temperature of 1°C. Incubation under these conditions was continued for 90 min, during which time their water and ionic content was distorted by loss of K^+ and gain of water, Na^+, Cl^- and Ca^{2+} (cf Deyrup, 1953, Mudge, 1951, Macknight, 1969). Fifteen minutes before the end of this period, the slices were transferred to Warburg manometric flasks, each containing 3 ml of the appropriate medium in the main vessel and 0.2 ml 5N-NaOH in the centre well. The vessels were attached to the manometers and gassed with O_2. They were then transferred to an incubation bath maintained at 25° or 38° for incubation for appropriate periods. During this incubation, the changes of composition which had occurred at 1°C were to a large extent reversed, the net movements of the ions into or out of the tissue during incubation providing a measure of the transporting activity of the tissue. Measurements of O_2 consumption were also made during this time by manometry. The basic incubation medium contained 146.5 mM Na^+, 5.0 mM K^+, 1.2 mM Ca^{2+}, 1.0 mM Mg^{2+}, 162.7 mM Cl^-, 1.0 mM SO_4^{2-} and Tris 10 mM (pH 7.0). Adjustment of this medium to pH was

made at the temperature at which it was to be used. Lead was added at the acetate or nitrate, with the corresponding Na^+ salts added to control slices. The maximal concentration of lead attainable in this medium at 1°C was 200 µM, and this, therefore, represents the upper limit of the concentration we have been able to study in vitro. After completion of the incubation, slices were collected for assay by tipping flask contents (after removal of the alkali from the centre wells with a roll of absorbent paper) onto a filter paper (Whatman No. 54) on a sintered glass funnel.

(b) Liver slices - Livers were sliced with a razor blade and glass slide as described above, according to the procedure of Elshove & van Rossum (1963). Incubation conditions and procedures were as described for kidney cortex slices. The liver slices were used mainly for the studies of tributyl-tin administered in vitro, the agent being added to the medium from a stock solution of 200 mM in Dimethyl sulphoxide; equivalent concentrations of the solvent were added to all flasks, including controls.

(c) Brain cortex - Rats were decapitated in a quillotine and the brain removed quickly and placed in chilled Krebs-Ringer Bicarbonate. The two brain hemispheres were separated by a blunt dissection through the corpus callosum and brain stem which was subsequently removed with curved forceps. Three slices of 0.35 ±0.15 mm thickness weighing 65-95 mg were obtained from each hemisphere (O'Neill et al., Biochem. Pharm. 23:809, 1963). Small cortical samples from each brain were frozen and saved for lead determinations.

Tissues were incubated for 30 minutes in a medium containing a low K^+ (5 mM) concentration, 6-^{14}C- and 6-^{3}H-Glucose under an atmosphere of 95% O_2 - 5% CO_2 in bicarbonate medium, pH - 7.4. At the end of 30 minutes the potassium ion concentration was raised to 105 mM and incubation continued for another 30 minutes. Following homogenization in 0.3 M perchloric acid and neutralization with 3M KOH-100 mM Imidazole buffer, the protein precipitate was removed and the tissue metabolites separated by the method of Bartlett as modified for brain by Duffy and O'Neill (Anal. Biochem. 24:132-140, 1968).

PREPARATION OF ISOLATED MITOCHONDRIA

(a) Kidney - Cortices were prepared as described above and mitochondria were then prepared from them by the method of Johnson & Lardy (1967), modified slightly as noted by van Rossum (1976), followed by two washings to remove EDTA which would otherwise chelate Pb^{2+} added in vitro. Respiratory activity was measured with a Clark-type O_2 electrode, as described in the latter work.

In some experiments, mitochondria were isolated from kidney cortex slices after incubation of the latter, in order to determine the proportion of the slice Ca^{2+} that was present in the mitochondria. The method used was that described above, except that a low concentration of La^{3+} was added in the homogenization medium to prevent uptake of Ca^{2+} by the mitochondria from other tissue components when the cells were initially disrupted. Justification for this method has been published by van Rossum et al., (1976).

(b) Brain Mitochondria - The procedure employed was essentially the method of Nicklas and Clark (1970) modified by Professor Salganicoff of this department. To obtain sufficient mitochondria it is necessary to use 8 rat brains. Each brain is obtained separately and within 30 seconds of decapitation the brain is placed in cold isolation medium and after removal of brain stem by blunt dissection with small curved forceps, chopped finely. To obtain satisfactory results, speed is essential to this point. The medium is then decanted and the minced tissue rinsed twice with chilled isolation medium (<5°C) to remove blood. The tissue is then transferred to a Dounce homogenizing tube (pestle clearance = .003 clearance) together with 40 ml of isolation medium; the tissue is then homogenized with 5 strokes of the pestle while cooled in an ice bath. The resulting homogenate is centrifuged at 2000 xg for 3 minutes. The supernatant is carefully decanted and saved, avoiding the "fuzzy layer." The crude pellet is resuspended in about 30 ml of isolation medium (leave blood clots behind) with 3 strokes of the homogenizer and centrifuged at 2000 x g for 3 minutes. After pooling the supernatants from the first and second steps, they were centrifuged for 8 minutes at 12,000 xg. The mitochondrial pellet thus obtained was resuspended in 10 ml of medium after removing a "fuzzy" overlayer. Five ml portions of this suspension were layered on ice-cold gradients consisting of 10 ml each of 3% and 6% Ficoll, respectively, and centrifuged at 8000 xg for 30 minutes in a swinging bucket rotor. Myelin and synaptosomal layers were decanted and the "fluffy layer" aspirated from the mitochondrial pellet. The mitochondrial pellet was finally suspended in an incubation medium minus EDTA (see below) and spun at 12,000 xg for 8 minutes. This step was repeated and the final pellet was suspended at 1-2 ml of incubation medium. These mitochondrial isolates are good for about 3 hours from the time of decapitation.

B. Melamed Modified L. Salganicoff Procedure

Isolation Medium	Incubation Medium
0.3M Mannitol	0.3M Mannitol
0.1% BSA	0.1% BSA
10 mM HEPES	10 mM HEPES pH 7.4
0.1 mM EDTA pH 7.4	

ANALYTICAL METHODS

A. Liver and Kidney

These were as described by van Rossum (1972) and van Rossum & Ernst (1978). Briefly, slices to be analyzed for water and ions were collected from the sintered glass filter into two tared weighing bottles, and their water contents and dry weights determined by drying for at least 4 hours at 110°C. All ions were extracted with 0.1 N-HNO_3, extraction being complete in about 4 hr (Little, 1964; van Rossum, 1970). Cations were analyzed on the atomic absorption spectrophotometer (Perkin-Elmer Model 303) using standard precautions to avoid interferences; Cl^- was estimated by electrometric titration (Aminco-Cotlove Titrator). Mitochondrial Ca^{2+} contents were analyzed by similar method.

In order to correct slice contents for extracellular water and ions, 0.5% (w/v) inulin was frequently included in the incubation media. Some of the slices from each incubation flask were then weighed wet and extracted with 20% (w/v) trichloracetic acid; inulin in the extract was assayed by the method of Kulka (1956).

For assay of adenine nucleotides, the slices were placed in 8% perchloric acid/40% ethanol and homogenized. After centrifugation, protein was estimated in the precipitate (Gornall et al., 1949) while the supernatant was neutralized and assayed for adenine nucleotides by the enzymic, fluorimetric methods of Williamson & Corkey (1969).

B. Brain Tissue

Tissues were treated and analyzed as described previously (O'Neill et al., J. Biol. Chem. 243:3345, 1970; Tjioe, Manian and O'Neill, J. Neurochem. 29:245, 1977; Sterling and O'Neill, J. Neurochem. 31:525, 1978.

FLUXES OF ^{45}Ca

Slices were "loaded" with ^{45}Ca during incubation at 1°C in the incubation medium to which 0.5 µCi ^{45}Ca/ml (specific activity approx. 0.55 µmol, in the final medium) had been added. Maximal loading was attained after 120 min, after which samples of slices were taken for analysis of total and radioactive Ca^{2+}. For determination of ^{45}Ca efflux curves, individual slices were washed in a series of beakers containing nonradioactive Ringer solution. Samples of the medium were counted after removal of the slices in order to determine the quantity of ^{45}Ca leaving the slice in each time interval. The intervals spent by each slice in a single beaker varied from 1 min during the first 3 min of efflux, to 30 min after the first 120 min of efflux. After the final washing period, the slices were collected for determination of their dry weight, total Ca^{2+} and ^{45}Ca. The slices were dried in the oven (see above) and ^{45}Ca extracted with 0.1 N-HNO_3. Radioactivity was determined in a liquid scintillation counter.

The efflux curves of ^{45}Ca were plotted semilogarithmically and analyzed graphically into exponential components as described by Solomon (1960). The intercept with the ordinate of the line representing each exponential was taken as an approximate estimate of the proportion of the initial ^{45}Ca which was present in the corresponding tissue "compartment." The validity of this procedure has been studied by Hagemeijer and van Remoortere (1969). From the proportions of ^{45}Ca in each compartment, and the total quantity of labeled Ca^{2+} present initially, the quantity of Ca^{2+} present in each "compartment" may be estimated in mmol/kg dry weight.

DETERMINATION OF MUSCARINIC BINDING IN DEVELOPING SPINAL CORD

Procedure for ^{3}H-QNB Binding

The binding studies employed high specific activity quinuclidinyl benzillate-tritium obtained from New England Nuclear Corporation, Boston. The nonradioactive quinuclidine benzillate was a gift of Dr. Leonard Cook, Roche

Laboratories, Nutley, New Jersey. The procedure was adapted for spinal cord from the method of Yamaura and Snyder (1974). The major modification involves the elimination of filtration on millipore filters and substitution of a high speed centrifugation at 18,000 rpm for 20 minutes.

(1) Excise spinal cords and place tissue in homogenizing tubes at 0°C.
(2) Add approximately 500λ NaK-PO_4 Buffer (pH 7.4) and homogenize tissue until you have a smooth suspension (15").
(3) Experimental set-up:

SAMPLE Tube #	VOLUME ^{3}H-QNB 0.16 mCi/µl	VOLUME QNB 2 X 10^{-7}M	VOLUME NaK-PO_4Buffer	VOLUME Protein Extract
1 & 2	10 µl	--	70 µl	5 µl
3 & 4	10 µl	--	70 µl	10 µl
5 & 6	10 µl	--	70 µl	20 µl
7 & 8	10 µl	5 µl	65 µl	5 µl
9 & 10	10 µl	5 µl	65 µl	10 µl
11 & 12	10 µl	5 µl	65 µl	20 µl
13	10 µl	--	70 µl	--
14	10 µl	5 µl	65 µl	--
15	--	--	70 µl	10 µl
16	--	5 µl	65 µl	10 µl

Tubes number 1 through 6 = Group I
Tubes number 7 through 12 = Group II
Tubes number 13 through 16 = Group III (Controls)

(4) Tubes 1 through 16 are then placed in a rack and allowed to stand at room temperature for 1 hour.
(5) Samples are placed in a microcentrifuge and spun at high speed for 1.5 minutes.
(6) Samples are then removed and placed in a Sorvall refrigerated centrifuge at 18,000 rpm and spun for 20 minutes.
(7) Tubes are placed in a rack and the supernatants are removed and discarded.
(8) The pellets in the tubes are then washed with 100 µl NaK-PO_4 Buffer, (vortex each sample about 15").
(9) Samples are then respun in the microcentrifuge for 1.5 minutes.
(10) Again samples are placed in the Sorvall and centrifuged at 18,000 rpm for 20 minutes.
(11) This washing procedure (steps 8,9,10) is repeated a second time.
(12) The tips of the test tubes containing the packed pellet samples are now cut from tubes using a clean scalpel blade. The pellet tips are then placed in labeled vials containing 10 ml of triton-toluene scintillation cocktail and tightly capped.
(13) The vials are shaken vigorously and placed in racks to stand at least 12 hours before counting.
(14) Vials to be used as correction blanks are prepared by cutting the tips from unused test tubes and placing them in scintillation vials in the same manner as was done in Steps 12 and 13.

COUNTING PROCEDURE

Dpms of samples are determined by using a channels ratio method. A series of seven quenched Tritium (^3H) standards was used to obtain a Quench Curve. This curve is necessary for the evaluation of the percent efficiency of the sample counts.

Calculations:

Part 1: CPM of all samples

gross CPM channel A
-average blk. CPM channel A
Actual CPM channel A

gross CPM channel B
-average blk. CPM channel B
Actual CPM channel B

Determine the ratio: CPM channel B/CPM channel A. Use this ratio to read out the percent efficiency from ^3H standard quench curve where B/A is the abcissa and percent efficiency is the ordinate.

Part 2: DPM of samples

$$\text{gross DPM of Samples} = \frac{\text{CPM A + CPM B}}{\text{Percent Efficiency}}$$

gross DPM Group I
-average DPM Group III
Actual DPM Group I

gross DPM Group II
-average DPM Group III
Actual DPM Group II

Group I = TOTAL BINDING DPM
Group II = NONSPECIFIC BINDING DPM
Group III = STANDARD (controls) DPM

Part 3: TOTAL BINDING ^3H-QNB (Group I)

$$\text{TOTAL MOLES } ^3\text{H-QNB} = \frac{\frac{\text{TOTAL DPM}}{2220}}{2.94 \times 10^{-13}}$$

Numbers 2220 and 2.94×10^{-13} are factors for specific activity and concentrations.

$$\frac{\text{TOTAL MOLES } ^3\text{H-QNB BOUND}}{\mu\text{g protein in ext'd sample}} = \text{Total moles bound per } \mu\text{g of protein.}$$

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Part 4: SPECIFIC BINDING ^{3}H-QNB (Groups I & II)

DPM Group I - DPM Group II = Specific Binding DPM

$$\text{SPECIFIC MOLES } ^3\text{H-QNB BOUND} = \frac{\frac{\text{Specific DPM}}{2220}}{2.94 \times 10^{-13}}$$

$$\frac{\text{SPECIFIC MOLES } ^3\text{H-QNB BOUND}}{\mu\text{g protein in ext'd sample}} = \text{SPECIFIC MOLES BOUND PER } \mu\text{g OF PROTEIN.}$$

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SECTION 5

RESULTS

EFFECTS OF LEAD IN VITRO

(a) Net movements of ions and water in kidney cortex slices - The derangement of tissue ion and water composition occurring during preincubation at 1°C (see Methods) and the subsequent restoration of composition during incubation under metabolically favorable conditions, are illustrated in Tables 1 and 2, the two sets of results differing in the temperature (38° and 25°C, respectively) used for the recovery period. When lead was added to the incubation media at 100 and 200 μM it had only two effects on the transport processes: (1) at 1°C, lead reduced the entry of Ca^{2+} into the tissue slices by about 30%; (2) the active reaccumulation of K^+ at 25° and 38°C was reduced, also by approximately 30%. Statistically significant effects were seen only at 200 μM Pb^{2+}. It will also be noted that the net extrusion of Ca^{2+} during recovery was also reduced by Pb^{2+}. However, this was a reflection of the lower initial level of Ca^{2+} (i.e., the level pertaining after incubation at 1°C) since the final Ca^{2+} content remaining in the slices was unaffected by Pb^{2+}. We conclude that Pb^{2+} reduced the quantity of extrudable Ca^{2+} initially present in the cells, but did not inhibit the mechanism for active extrusion of Ca^{2+}.

In view of the similarity of these results at the two recovery temperatures, subsequent experiments used 25°C for recovery since the tissue retains a better ultrastructural appearance and higher ATP levels at this cooler temperature.

The time-course of the net active movements of the ions was studied in the presence and absence of 200 μM Pb^{2+} in view of the possibility that short-term effects had been missed during the prolonged (60 min) incubation used for the recovery period in Tables 1 and 2. Fig. 1, however, shows that the only effect of Pb^{2+} was on the reaccumulation of K^+, and that this became evident only after 30 min at 25°C.

(b) Energy metabolism in kidney cortex slices - Table 2 includes measurements of the rate of respiration at 25°C of the slices discussed above, showing that 200 μM Pb^{2+} reduced O_2 consumption by 25%. In a separate series of experiments, the ATP contents of the slices at the end of the recovery period were assayed. Both respiration and ATP contents were lowered by a maximum of 30% at 200 μM Pb^{2+}, although lower concentrations also partially reduced these parameters (Fig. 2). The rate of O_2 consumption at a given concentration of Pb^{2+} was constant throughout the incubation period, suggesting that the rate

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TABLE 1. EFFECTS OF LEAD ON THE NET ION MOVEMENTS AND ATP CONTENT IN SLICES OF KIDNEY CORTEX FROM CONTROL RATS

	Fresh Tissue	90 min at 1°C			+ 60 min at 38°C		
Pb^{2+} added (μM)	--	0	100	200	0	100	200
Water kg/kg dry wt	3.57 ±0.05	4.26 ±0.16	4.17 ±0.07	4.24 ±0.24	3.34 ±0.13	3.34 ±0.08	3.14 ±0.04
Na^+ mmol/kg dry wt	310 ± 3	713 ± 6	681 ± 9	683 ± 4	490 ± 6	488 ± 7	463 ± 3
Cl^-	355 ± 5	755 ± 23	-- ±	703 ± 17	638 ± 8	-- ±	566 ± 18
K^+	360 ± 12	119 ± 1	112 ± 2	109 ± 1	210 ± 2	187 ± 2	173 ± 2
Ca^{2+}	8.3 ±0.3	34.7 ± 1.3	28.3 ± 1.3	23.8 ± 0.7	13.7 ± 0.9	14.2 ± 0.7	14.3 ± 0.4

Mg^{2+} were not significantly affected by incubation or by the treatment with lead, and are therefore not shown.

TABLE 2. EFFECTS OF LEAD ON NET MOVEMENTS OF ION AND WATER AND ON RESPIRATION AND ATP IN SLICES OF KIDNEY CORTEX FROM CONTROL RATS DURING INCUBATION AT 1° AND 25°C.

	90 min at 1°C			+ 60 min at 25°C		
Pb^{2+} added (μM)	0	100	200	0	100	200
Water kg/kg dry wt.	2.46 ±0.04	2.34 ±0.26	2.48 ±0.06	1.59 ±0.09	1.74 ±0.12	1.63 ±0.04
Na^+ mmol/kg dry wt.	290 ± 23	243 ± 17	249 ± 36	80 ±12	134 ± 22	92 ±19
Cl^-	312 ± 16	321 ± 2	327 ±31	150 ± 14	209 ± 19	168 ± 14
K^+	121 ± 4	125 ± 8	131 ± 5	302 ± 16	278 ± 13	247* ± 9
Ca^{2+}	25.8 ± 2.9	22.2	13.3* ± 0.3	7.7 ±0.6	10.3 ± 1.1	8.3 ±0.8
O_2 uptake μl/mg dry wt/h	--	--	--	5.92 ±0.15	5.35 ±0.15	4.53* ±0.22

The water and ion values refer to intracellular contents, calculated on the assumption that the inulin-containing water compartment of the slices had the same ionic composition as the incubation medium. Values are mean ± SEM of 3 observations at 1°C and of 10-12 observations at 25°C.

*Significantly different from value without lead ($p < 0.01$).

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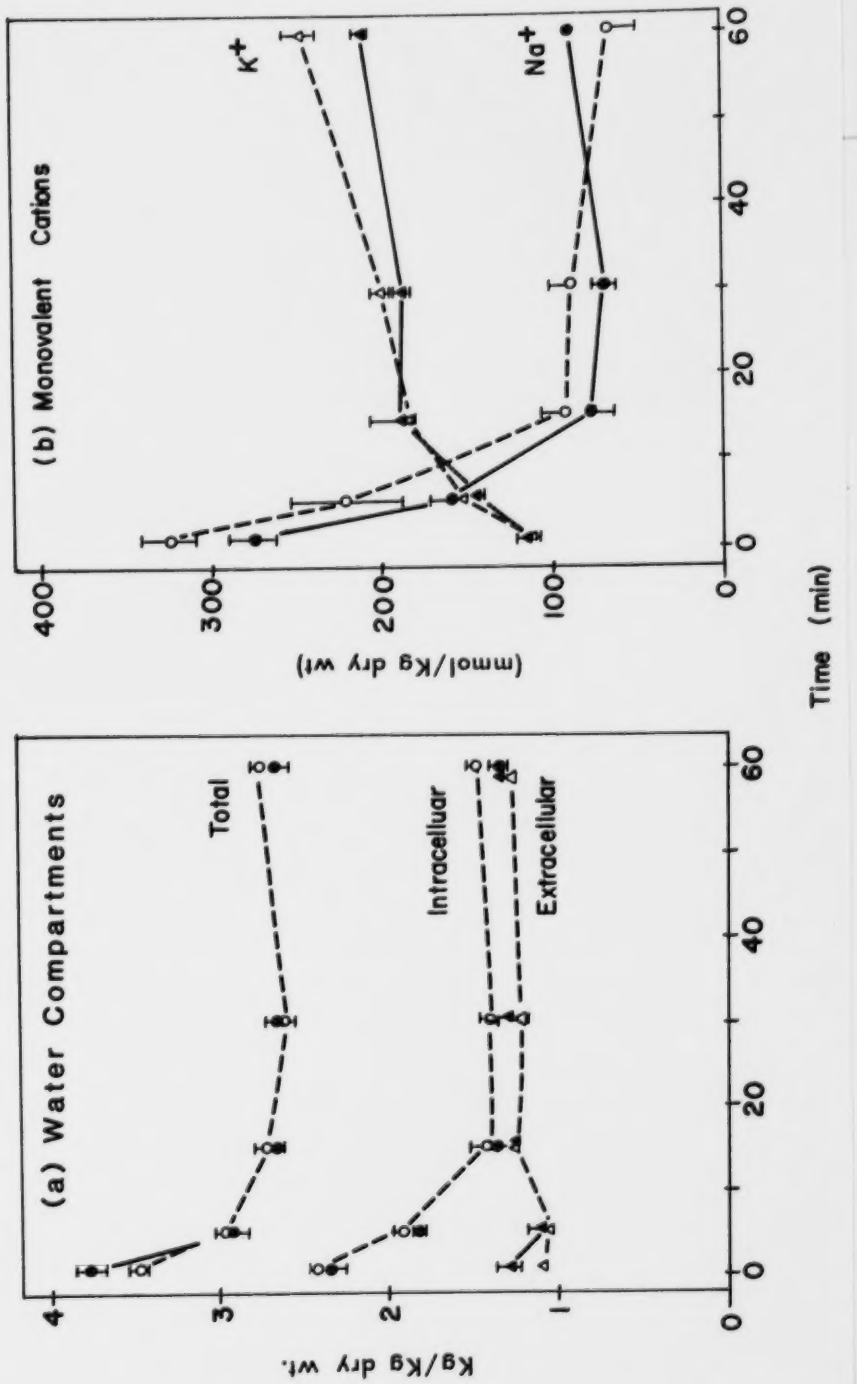

Figure 1a. Water Compartments; Figure 1b. Monovalent Cations.

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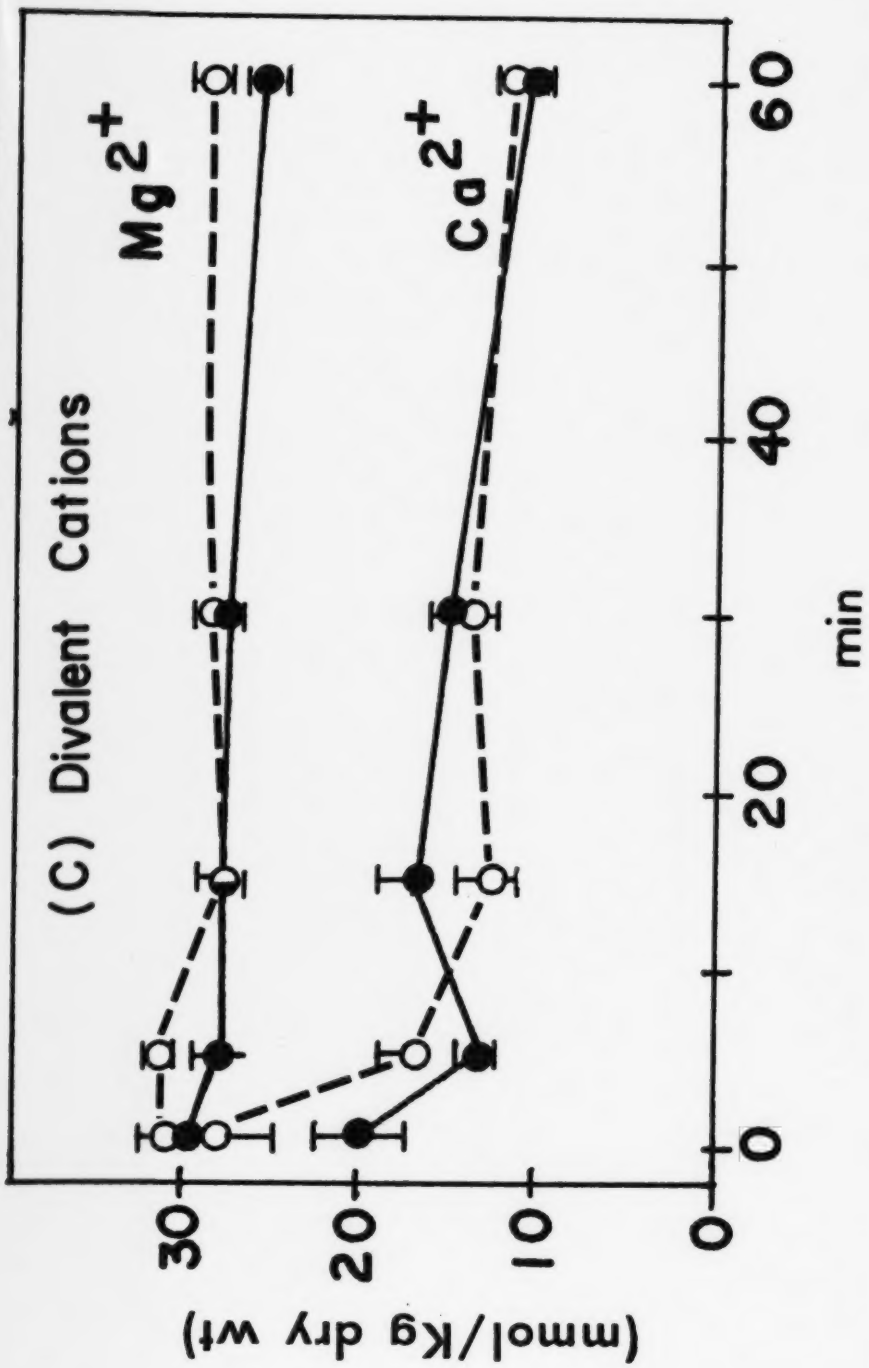

Figure 1c. Divalent Cations.

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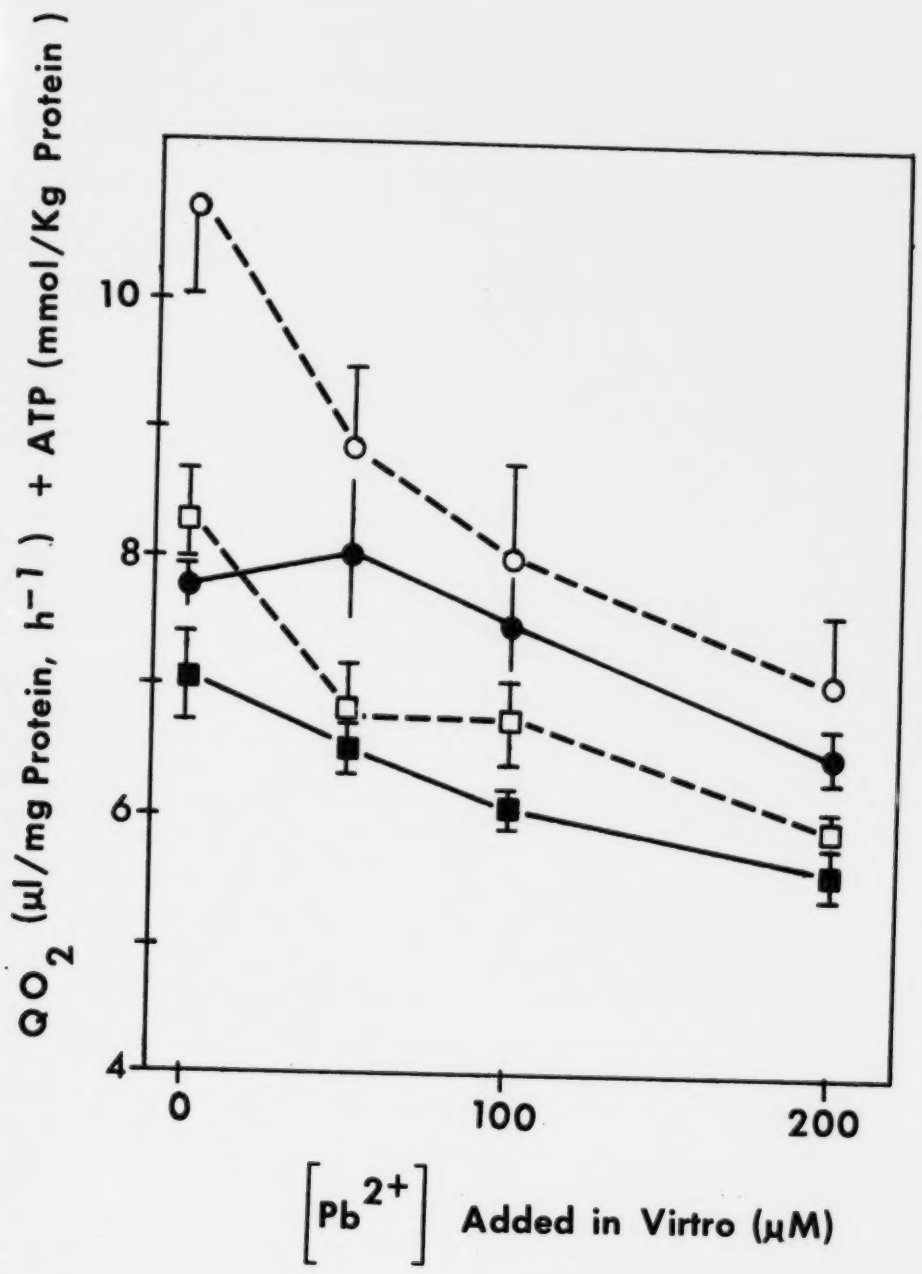

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Figure 2. Effect of Pb Concentration in vitro on QO_2 and ATP.

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of ATP synthesis was probably also constant. However, Fig. 2b shows that the ATP content of the slices underwent a net increase during the first 15-30 min at 25°C, after which a steady state was attained in the control slices. In the presence of 200 μM Pb^{2+}, the ATP content was identical to control levels at 15 min, but thereafter fell slightly below the controls. The effects of Pb^{2+} on ATP contents was apparent only after at least 30 min, a finding reminiscent of the effect of Pb^{2+} on K^+ reaccumulation (Fig. 1a). Similar results were obtained at 38°C, but the ATP levels were lower; e.g., at 38°C control slices contained 3.9 ± 0.2 mmol/kg protein after 60 min, while slices at 25°C contained 5.9 ± 0.1 mmol/kg.

(c) Possible interpretations and related experiments - At least two interpretations of the effects of Pb^{2+} on active transport of K^+ are possible from known effects of the heavy metal on subcellular systems: (1) Pb^{2+} acted by a specific inhibition of the Na-K-ATPase (Nechay & Saunders 1978), the inhibition of energy metabolism being a secondary effect which reduced the ATP by too small an extent to affect the activity of the transport system. In this case, the continuing transport of Na^+ (which was not affected by Pb^{2+}) would be due to a compensating action of the "second Na^+ pump" which is not dependent on the Na-K-ATPase (Proverbio et al., 1970). (2) The primary effect of Pb^{2+} was inhibition of mitochondrial energy conservation, the partial inhibition of K^+ transport resulting from limitation of the ATP needed for the Na-K-ATPase system.

We have attempted to distinguish between these two possibilities by making a comparison of the above results with the effects of a specific inhibitor of the Na-K-ATPase, ouabain, and a specific inhibitor of mitochondrial electron transfer, Antimycin A. In each case we were concerned to see the relationship between K^+ uptake, Na^+ extrusion, ATP levels, and respiration at the concentration of inhibitor which gave the same reduction (approximately 30%) of K^+ accumulation as that given by 200 μM Pb^{2+}. Accordingly, we carried out titrations of the tissue slices with concentrations of each inhibitor.

At a concentration of 1 μg/ml, Antimycin A gave a 40% reduction of K^+ uptake which was accompanied by a 30% reduction of respiration and 37% fall of ATP content - results similar to the effects of 200 μM Pb^{2+} (Fig. 3). However, unlike the effect of Pb^{2+}, there was a significant, 20% reduction of Na^+ extrusion.

In the case of ouabain, a concentration of 200 μM mimicked 200 μM Pb^{2+} in causing a 30% reduction of K^+ uptake, 25% reduction of respiration and no significant inhibition of Na^+ extrusion (Fig. 4). It did not reduce ATP levels, but this is consistent with the concept that ouabain lowers energy metabolism because of the "pacemaker" effect of ion transport on oxidative phosphorylation (Whittam, 1962).

These results, while extending our understanding of the overall situation when different means are used to achieve a 30% inhibition of K^+ transport, do not allow a clear distinction to be made between the two possible effects of Pb^{2+} outlined above. The action of lead on the two transport processes (i.e., of Na^+ and K^+) is more similar to the effects of ouabain than of Antimycin A,

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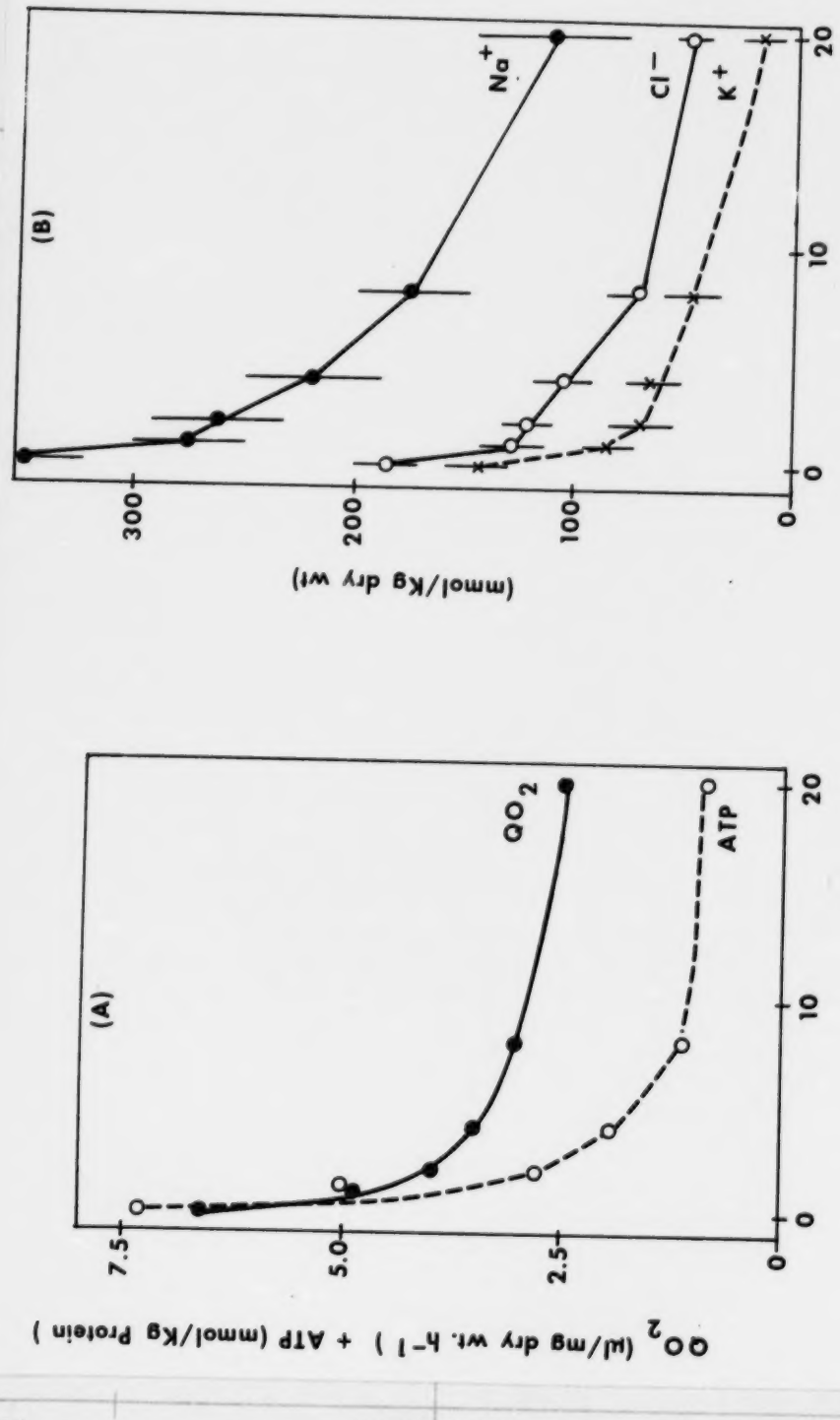

Figure 3a. QO_2; ATP; Figure 3b. Na^+, Cl^+, K^+.

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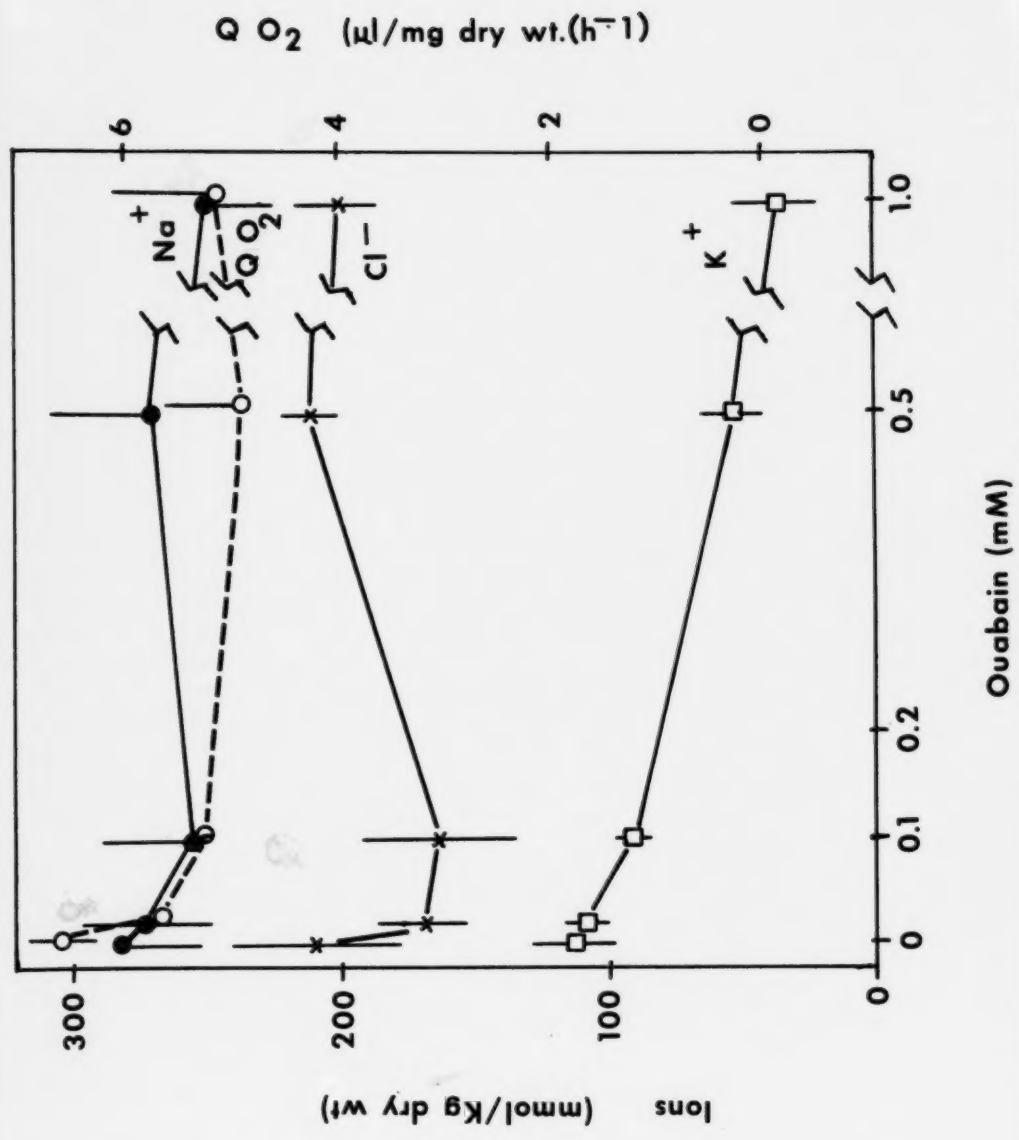

Figure 4. Effect of Ouabain on QO_2, Na^+, K^+ and Cl^-.

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but it is then difficult to explain why the 30% reduction of ATP caused by Pb^{2+} should not, like that caused by Antimycin A, inhibit Na^+ transport as well as K^+ transport. The similarity of the time-course of the effects of lead on K^+ transport and ATP levels provides extra, circumstantial evidence favoring limitation of high-energy compounds as the cause of transport inhibition but, again, we are unable to account for the failure to see inhibition of Na^+ transport also.

(d) Respiration of isolated mitochondria from kidney cortex - One possible way to account for the above discrepancy might be that Pb^{2+} did not reduce ATP in the slices by way of an inhibition of oxidative phosphorylation in the mitochondria, but by another means. We, therefore, examined the effects of Pb^{2+} *in vitro* on the oxidative metabolism of mitochondria isolated from the kidney cortex, since previous work indicating an inhibitory effect of Pb^{2+} on this activity of the cortex only made use of lead treatment *in vivo* (Goyer & Krall, 1969). However, studies with the O_2 electrode, using different substrates, show that Pb^{2+} *in vitro* does in fact affect mitochondria.

Typical electrode traces are shown in Fig.5a,b and the mean results from a series of experiments of the type illustrated in Fig.5a,b are summarized in Fig. 6. With pyruvate + malate as the substrate, Pb^{2+} inhibited respiration in State III (phosphate acceptor present; see Chance & Williams, 1955) and also reduced the respiratory control ratio, implying a reduction of phosphorylating activity. Half-maximal effects were given on State III respiration by 30 µM Pb^{2+} and on the respiratory control ratio by 20 µM. Succinate as substrate showed similar responses to Pb^{2+} although the more marked effect was an increase in the rate of State IV respiration (no acceptor).

We conclude that the reduction of tissue ATP levels caused by Pb^{2+} *in vitro* is most probably due to a direct inhibition of ATP production in the mitochondria.

(e) Ca^{2+} contents of kidney cortex slices incubated at 1°C - The second effect of Pb^{2+} on ion exchanges in the slices of kidney cortex, namely the reduction of Ca^{2+} uptake at 1°C, was studied in two ways: (1) Mitochondrial Ca^{2+} - The extent to which Ca^{2+} entered the mitochondrial compartment within the slices was expected to present a possible basis for the effect of Pb^{2+} in reducing the total entry of Ca^{2+} at 1°C, since we have previously shown that Ca^{2+} entering liver slices under similar conditions partially enters the mitochondria (van Rossum et al., 1976), and since Parr & Harris (1976) have shown that Pb^{2+} inhibits Ca^{2+} uptake by mitochondria isolated from heart. To investigate this likelihood, we isolated mitochondria from kidney cortex slices before and after incubation at 1°C. La^{3+} was added to the homogenization medium to prevent mitochondria taking up Ca^{2+} from nonmitochondrial sources upon disruption of the cells. Table 3 shows that Ca^{2+} entering the mitochondria contributed about 40% of the total uptake by the slices at 1°C. The quantity of Ca^{2+} entering the mitochondria was substantially reduced in slices which had been incubated in the presence of Pb^{2+}, a number of different salts of this heavy metal giving similar results.

In the absence of Pb^{2+}, the entry of Ca^{2+} into the mitochondria was inhibited by inhibitors of mitochondrial electron transfer, especially cyanide,

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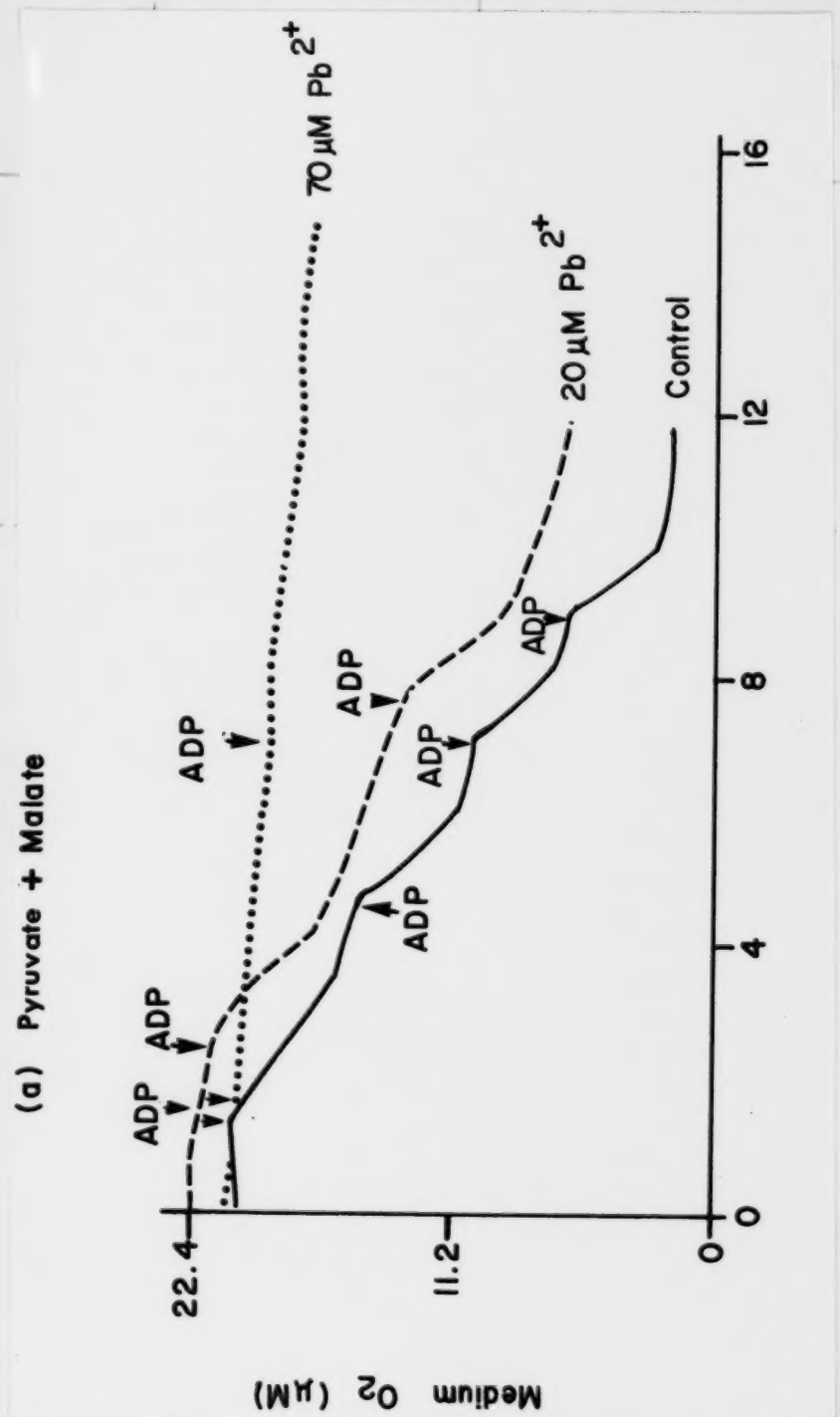

Figure 5a. Effect of Pb^{++} (20 & 70 μM) on Pyruvate and Malate.

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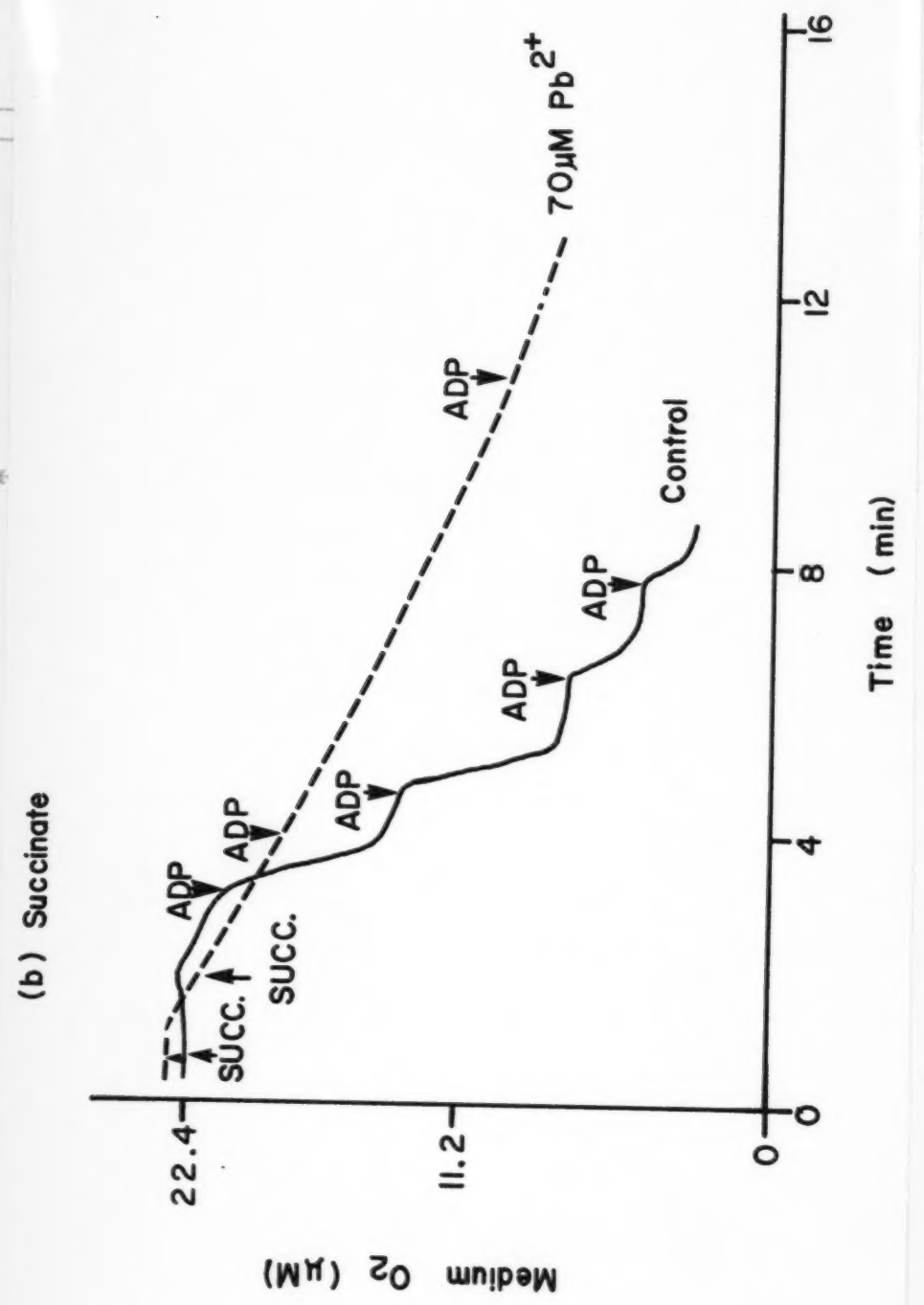

Figure 5b. Effect of Pb++ on Succinate.

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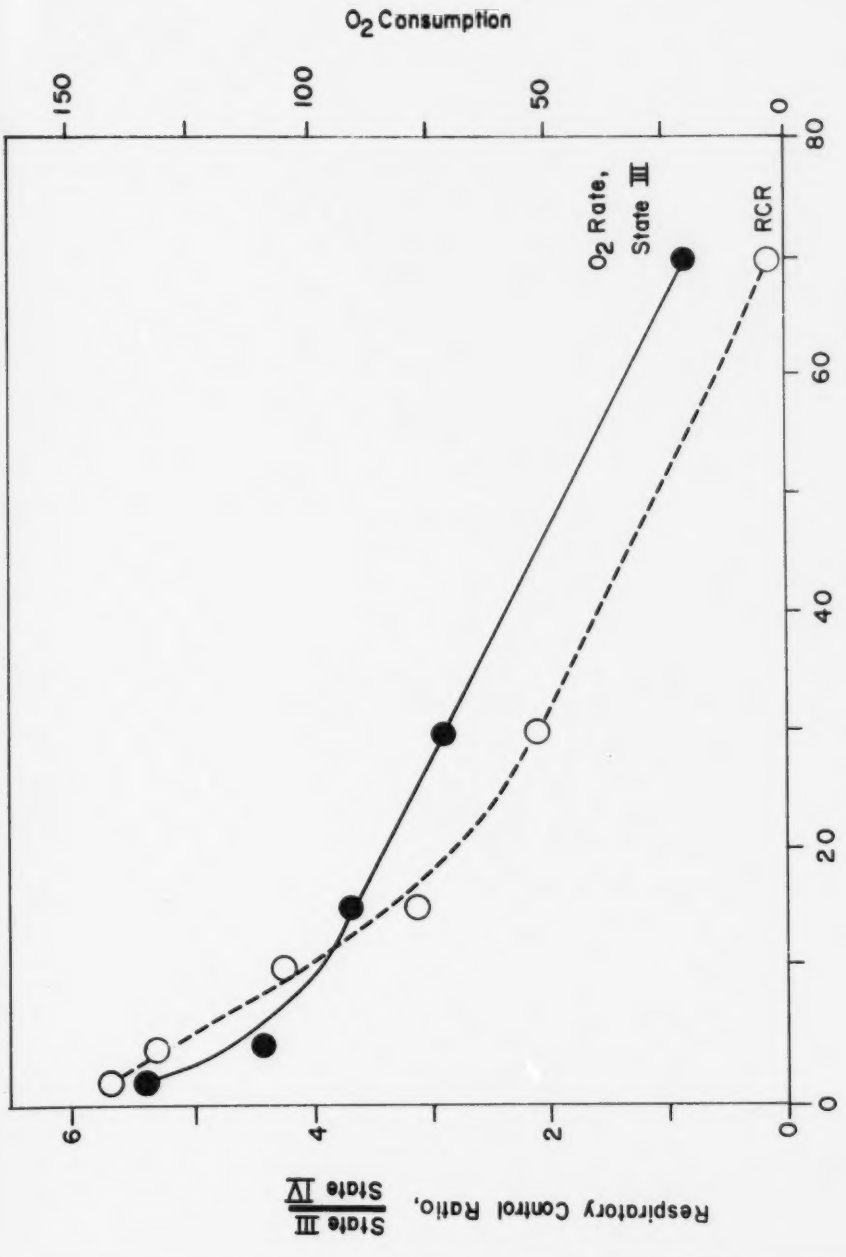

Figure 6. Effect of Pb^{++} Concentration on QO_2 and RCR.

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TABLE 3. Ca^{2+} CONTENT OF MITOCHONDRIA ISOLATED FROM KIDNEY-CORTEX SLICES AFTER INCUBATION OF THE SLICES FOR 90 MIN AT 1°C IN TRIS-BUFFERED RINGER SOLUTION, IN THE PRESENCE OF LEAD SALTS AND INHIBITORS.

	Ca^{2+} Contents					
	Slices		Mitochondria			
Additions to Medium	mmol/kg slice dry wt		mmol/kg mitoch. protein		mmol/kg slice dry wt	
None	31.2	1.8	48.0	1.2	12.1	0.2
Sodium Acetate	29.5	2.3	44.3	1.2	11.9	0.1
Lead Acetate	21.2	1.9	28.5	0.7	8.3	0.2
$Pb(NO_3)_2$	20.1	2.1	33.7	1.4	8.5	0.2
NaCN	22.3	1.9	29.9	1.3	7.0	0.3
Antimycin A	26.6	2.6	41.8	1.7	10.1	0.2

Lead salts and sodium acetate were present at 200 μM, NaCN at 2 mM and antimycin A at 25 mg/ml.

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implying that the Ca^{2+} uptake into these organelles was due to an active accumulation mechanism. That the accumulation of Ca^{2+} by mitochondria remains active at 1°C has been noted by Chance (1965).

Compartmental analysis of Ca^{2+} in the slices at 1°C - To obtain another indication of the effect of Pb^{2+} on the distribution of Ca^{2+} in kidney cortex slices after incubation at 1°C, the exchangeability of tissue Ca^{2+} with ^{45}Ca in the medium, and the efflux of ^{45}Ca from the tissue after attainment of a steady state, were studied.

Table 4 shows that the specific activity of ^{45}Ca attained by tissue Ca^{2+} after 90 min incubation in a medium containing this radioisotope at 1°C was about 70% of the specific activity of the medium. This represents the fraction of the tissue calcium which is able to exchange with Ca^{2+} of the medium; the value we have obtained is similar to that found by Höfer & Kleinzeller (1963) for rabbit kidney cortex. Lead (200 μM) had no significant effect on the fraction of Ca^{2+} that was thus found to be exchangeable, but calculation of the quantity of Ca^{2+} per kg dry weight in the exchangeable and nonexchangeable "compartments" showed that both were reduced in the presence of Pb^{2+} (Table 4).

The distribution of the exchangeable Ca^{2+} into kinetically distinguishable compartments was determined by studying the efflux of ^{45}Ca into aliquots of nonradioactive medium. A composite curve of 14 experiments is illustrated in Fig. 7. During the early phases (up to 50 min) efflux was more rapid in the presence of Pb^{2+}, while efflux in the slowest phase was at a similar rate in the presence or absence of Pb^{2+} (i.e., from 50 min onwards in Fig. 7). The results of compartmental analysis are included in Table 4. It can be seen that while Pb^{2+} altered the percentage of tissue Ca^{2+} in the two slowest components of the efflux curve, the Ca^{2+} in the slowest of the three components was the only one to be altered when expressed as mmoles of Ca^{2+} per kg dry weight. Kinetic components are not readily equitable with structural components of the tissue, but it may be tentatively suggested that this most slowly effluxing component contains a considerable component of the mitochondrial Ca^{2+}, as Höfer & Kleinzeller (1963) found it to be increased in efflux rate by addition of the mitochondrial uncoupler, 2,4-dinitrophenol. The effect of Pb^{2+} on the quantity of Ca^{2+} in this component (i.e., a reduction of approximately 4 mmol Ca^{2+}/kg slice dry weight) is similar to its effect on mitochondrial Ca^{2+} determined directly (cf. Tables 3 and 4).

The nature of the nonexchangeable Ca^{2+}, the only other component to be affected by Pb^{2+} remains to be elucidated, but it may tentatively be suggested to be Ca^{2+} bound to negatively charged tissue structures which can be displaced by binding of Pb^{2+} to the same sites.

Effects of Pb^{++} on Brain Mitochondria

Brain mitochondria have been found to be sensitive to lead added *in vitro*. Earlier studies by Holtzman & Hsu (1976) clearly showed that brain mitochondrial respiratory control was adversely affected by the presence of lead added *in vitro*. However, there was no detectable effect below 50 μM. This would suggest that at levels found in brain *in vivo* by this laboratory and others

TABLE 4. COMPARTMENTALIZATION OF Ca^{2+} IN SLICES OF KIDNEY CORTEX INCUBATED AT 1°C, STUDIED BY INFLUX AND EFFLUX OF ^{45}Ca.

"Compartment"	Ca^{2+} in "compartment" as % of total tissue Ca^{2+}		Ca^{2+} in "compartment" mmol/kg slice dry wt			
	Control	+ Pb^{2+}	Control		Pb^{2+}	
Total slice Ca^{2+}	100	100	29.5	2.3	20.1	1.7
Nonexchangeable	29.9	23.4	8.0	0.8	4.5	0.3
Exchangeable-Total	70.1	76.6	20.6	1.1	15.6	0.9
I*	14.6	15.2	4.3		3.1	
II	24.9	36.8	7.3		7.5	
III	30.6	24.6	9.0		5.0	

*Compartments I-III are the kinetically distinguishable components of the efflux curve of ^{45}Ca, representing subdivisions of the total exchangeable Ca^{2+}. Compartment I was the most rapid efflux component, compartment III the slowest.

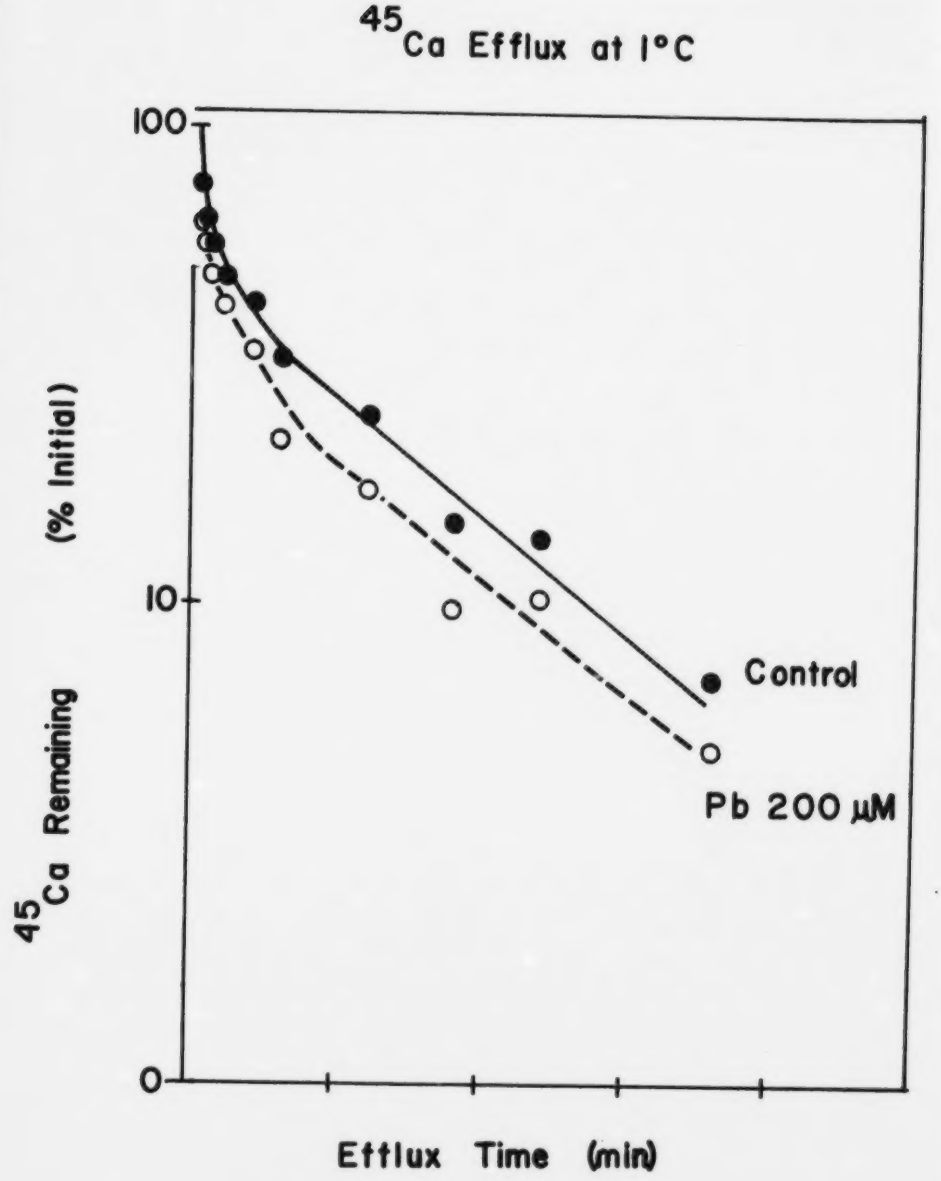

Figure 7. Effect of Pb^{++} (200 mM) on Ca^{++} Efflux at 1°C.

(circum 0.5 - 5 μM) would not be sufficient to affect oxidative energy metabolism. In studies to be reported (O'Neill, et al., Abstr. #1397, Soc. for Neurosci. 5:413, 1979) we have found inhibitory effects of lead acetate over this lower concentration range during State III (Plus ADP) respiration with either pyruvate (10 mM) plus malate (2 mM) or with succinate (10 mM). Many effects of lead appear to be competitive with calcium among these being chelated by ethylenediaminetetracetic acid (EDTA). The buffering action of EDTA interferes with the inhibitory effects of lead ions. It is suggested that the presence of EDTA probably accounts for the failure by Holzman to demonstrate lead effects below 50 μM. These results are preliminary in nature but they do appear to be consistent with the more extensive studies on liver and kidney mitochondria. Typical Clarke-electrode tracings are shown (Fig. 17 a,b).

EFFECTS OF LEAD IN VIVO

For comparison with the effects of Pb^{2+} *in vitro* described in the preceding section, we have made some studies of the effects of administering Pb^{2+} *in vivo* according to the schedule described under "Methods." We have used two approaches to determine any effects: (1) measurement of the ionic composition of kidney cortex tissue taken directly after death of treated and control animals; (2) by preparing slices of the cortex from treated and control animals and carrying out experiments on their ion-transporting and energy-conserving metabolism, of the type used for studying the effects of Pb^{2+} *in vitro*.

(a) Lead treatment *in vivo* was found to have no significant effect on the ion and water contents of kidney cortex *in vivo* (not illustrated). It is possible that longer treatments with lead might be needed for an effect to become evident in the living animal, and we are currently engaged in a series of such experiments.

(b) The ability of slices of kidney cortex to restore their ion and water contents after incubation at 1°C was also unimpaired after treatment with Pb^{2+} *in vivo*. However, the slices showed a rate of respiration which was 15% less, and ATP contents which were 30% less, when the slices were prepared from lead-treated rats than slices prepared from control animals (Fig. 2 - points at abscissa value of "zero Pb^{2+}"). When the incubation *in vitro* was conducted in the presence of 40-200 μM Pb^{2+}, the values of respiration and ATP showed further decreases (Fig. 2), but these were such that the minimum levels pertaining to the slices from lead-treated rats did not differ significantly from the levels in slices from control rats (Fig. 2 - points at abscissa value of "200 μM Pb^{2+}"). Thus, the effects of lead treatment *in vivo* and *in vitro* were not additive, and we tentatively conclude that, at least as far as respiratory energy metabolism is concerned, lead *in vivo* acts predominantly at the same site as it does *in vitro*.

BRAIN ACETYLCHOLINE SYNTHESIS

The pathway of glucose metabolism leading to the synthesis of acetylcholine has been traced employing a mixture of tritium (^{3}H) and carbon-^{14} labeled glucose (Sterling and O'Neill, 1978). Depicted in Fig. 8 are two pathways leading from glucose to acetylcholine with pyruvate as a common intermediate.

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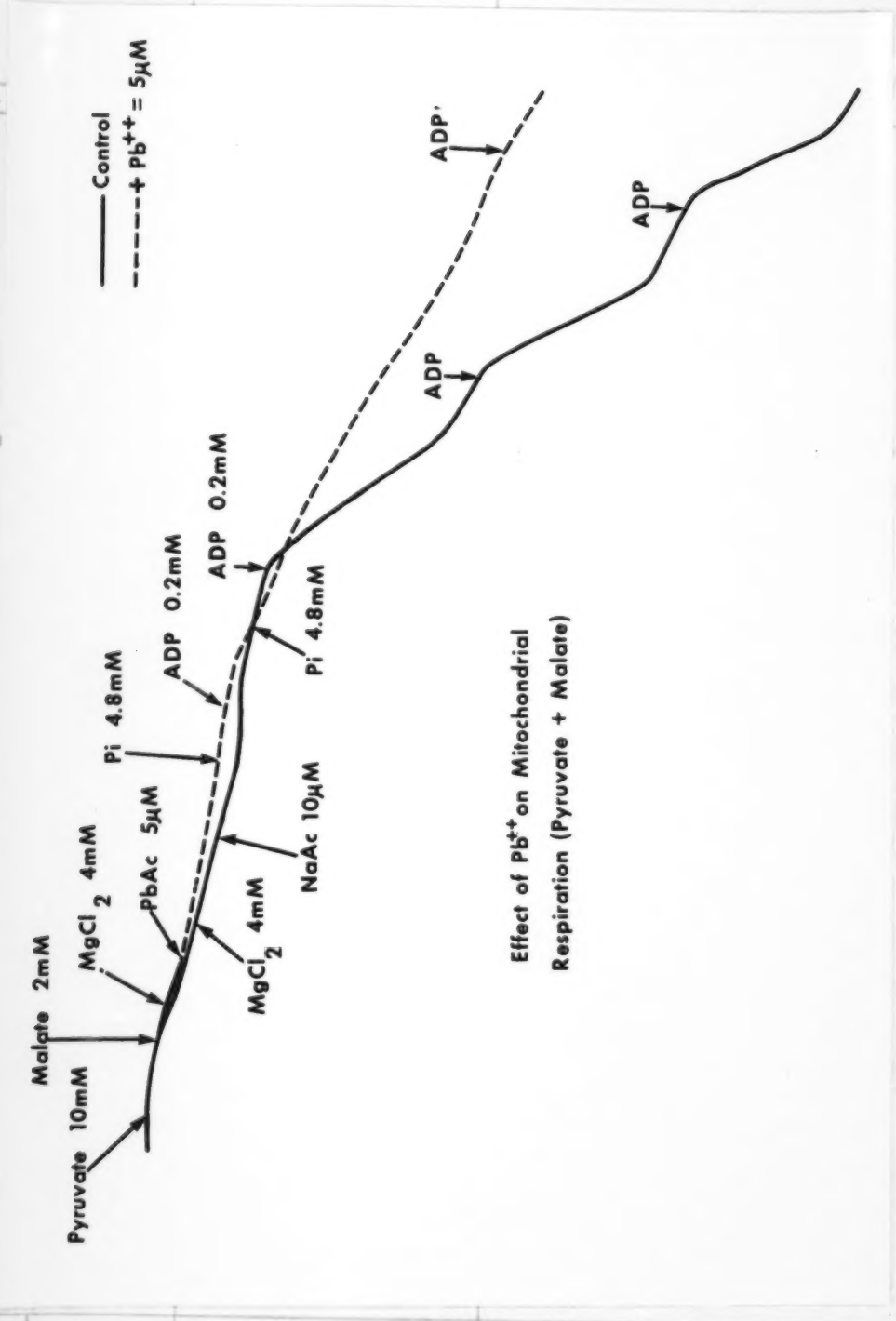

Figure 17a. Effect of Pb^{++} on mitochondrial respiration (Pyruvate + Malate.

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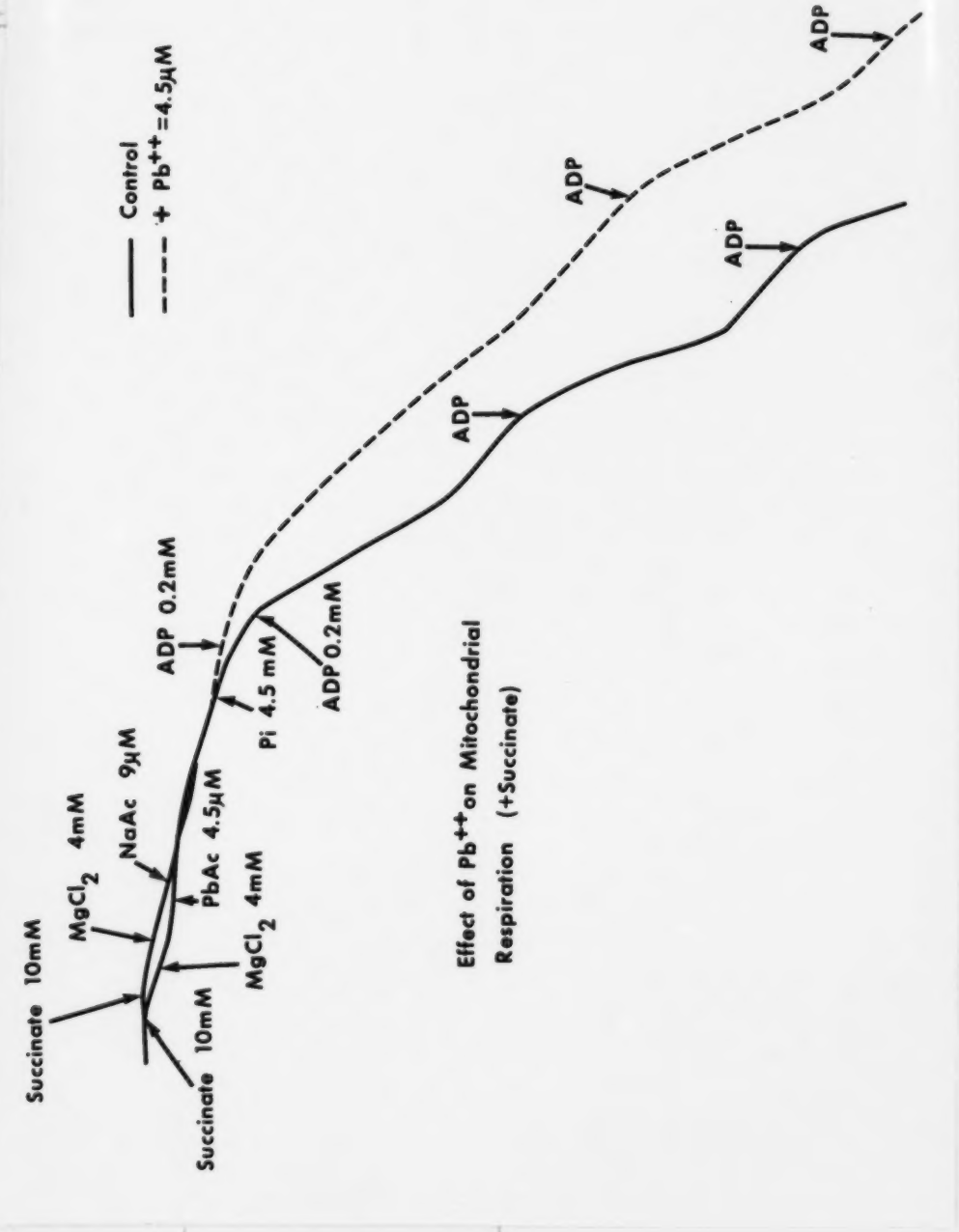

Figure 17b. Effect of Pb++ on mitochondrial respiration (+ succinate).

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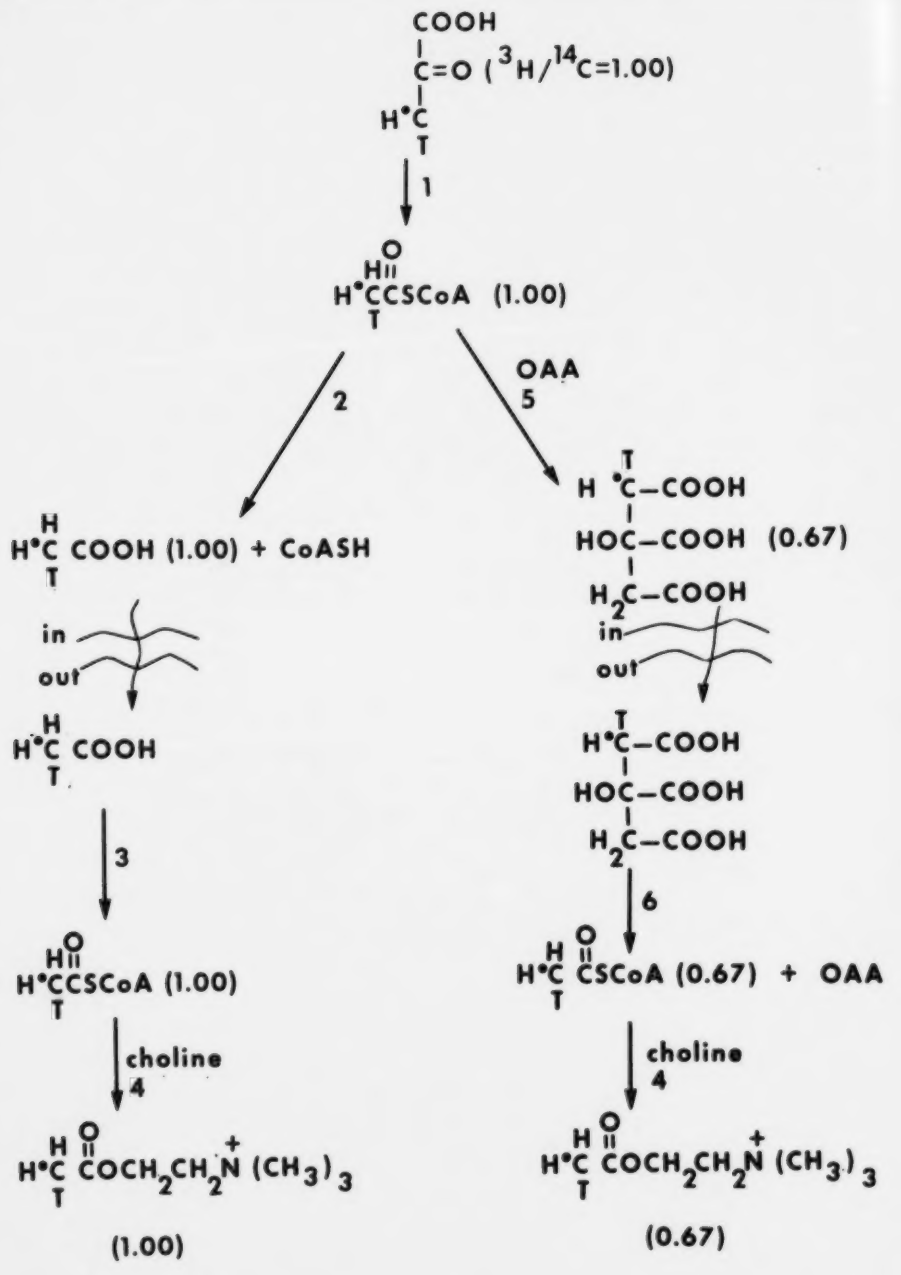

Figure 8. Alternate pathways leading to acetylcholine synthesis.

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Starting with a $^{3}H/^{14}C$ ratio equal to 1, the $^{3}H/^{14}C$ ratio at the level of pyruvate (or lactate) is 1. If pyruvate is converted to acetyl CoA via acetate, the $^{3}H/^{14}C$ ratio in acetylcholine would also be 1.0. If alternatively pyruvate is first converted to citrate in the mitochondrion there would be a loss of tritium in citrate ($C^{-4,5}$) to 2/3 (0.67) hence, the resulting acetyl-CoA would have a $^{3}H/^{14}C = 0.67$ as would the final product, acetylcholine(cf.Figs.9,10,11).

Shown in Table 5 are data for lactate, citrate ($C^{-4,5}$) and citrate (total) malate and acetylcholine. The salient features to be noted are that despite the large amount of radioactivity in lactate, the $^{3}H/14C$ ratio remains essentially unchanged. In contrast citrate (total) has a ratio of 0.37 but the carbons 4,5 retain a ratio of 0.65. This is significant because it is these 2 carbons when citrate is cleaved by ATP-CoA-citrate lyase that form acetyl-CoA in the cytosol. This accounts for the ratio of $^{3}H/^{14}C = 0.71$ in acetylcholine.

In Table 6 are presented data obtained from brains of adult male rats exposed for twenty(20) days to Pb^{++} (600 ppm) in drinking water. In our progress report (dated April 1, 1977 - March 31, 1978), we had indicated that acetylcholine synthesis was inhibited over the same twenty(20) day period at 200 ppm Pb^{++} but there did not appear to be an inhibition of glycolysis as measured by lactate production. In contrast, at 600 ppm Pb^{++} there was a considerable decrease in lactate production, and citrate levels as well as an inhibition of acetylcholine synthesis.

Evidence is accumulating which indicates that when the citrate pathway is interfered with (Sterling and O'Neill, 1978) acetoacetate can serve as the acetyl-donor for acetylcholine synthesis. In Table 7 control data are provided to describe this pathway employing ($3-^{14}C$) β-hydroxybutyrate as an acetoacetate precursor. The $^{3}H/^{14}C$ ratio in lactate is high (57.1 ± 4.2) because little carbon-14 is incorporated. A significant amount of carbon enters the tricarboxcyllic pathway and accounts for the ratio $^{3}H/^{14}C = 0.27$ in citrate ($C^{-4,5}$) but an even high ratio in acetylcholine. This would be expected since acetoacetate formed intramitochondrially from β-OH butyrate enters the cytosol and is cleaved by a thiolase to acetyl-CoA. The latter enters acetylcholine from the acetyl-CoA pool.

In Table 8 again we see a high $^{3}H/^{14}C$ ratio in lactate (55 ± 2.7) but a 22% decrease in synthesis. Similarly, there was observed a 50% decrease in acetylcholine although the ratio $^{3}H/^{14}C$ did not appear to be significantly affected (0.44 ± .03 vs 0.37 ± .03 for the control). Since there was approximately the same amount of ACh inhibition, whether the citrate pathway or the acetoacetate pathway was measured, it appears that lead is exerting some generalized effect, probably on energy metabolism.

RESULTS OF ELECTRON MICROSCOPY STUDIES

A series of studies has been performed on pregnant control animals and pregnant animals exposed to 200 ppm lead in drinking water during this period and throughout the subsequent weaning period. Spinal cords of fetuses 16 days and older and postnatal animals were fixed in glutaraldehyde (2.5% - 2% osmium tetroxide) for transmission EM. In order to better assess whether lead-expo-

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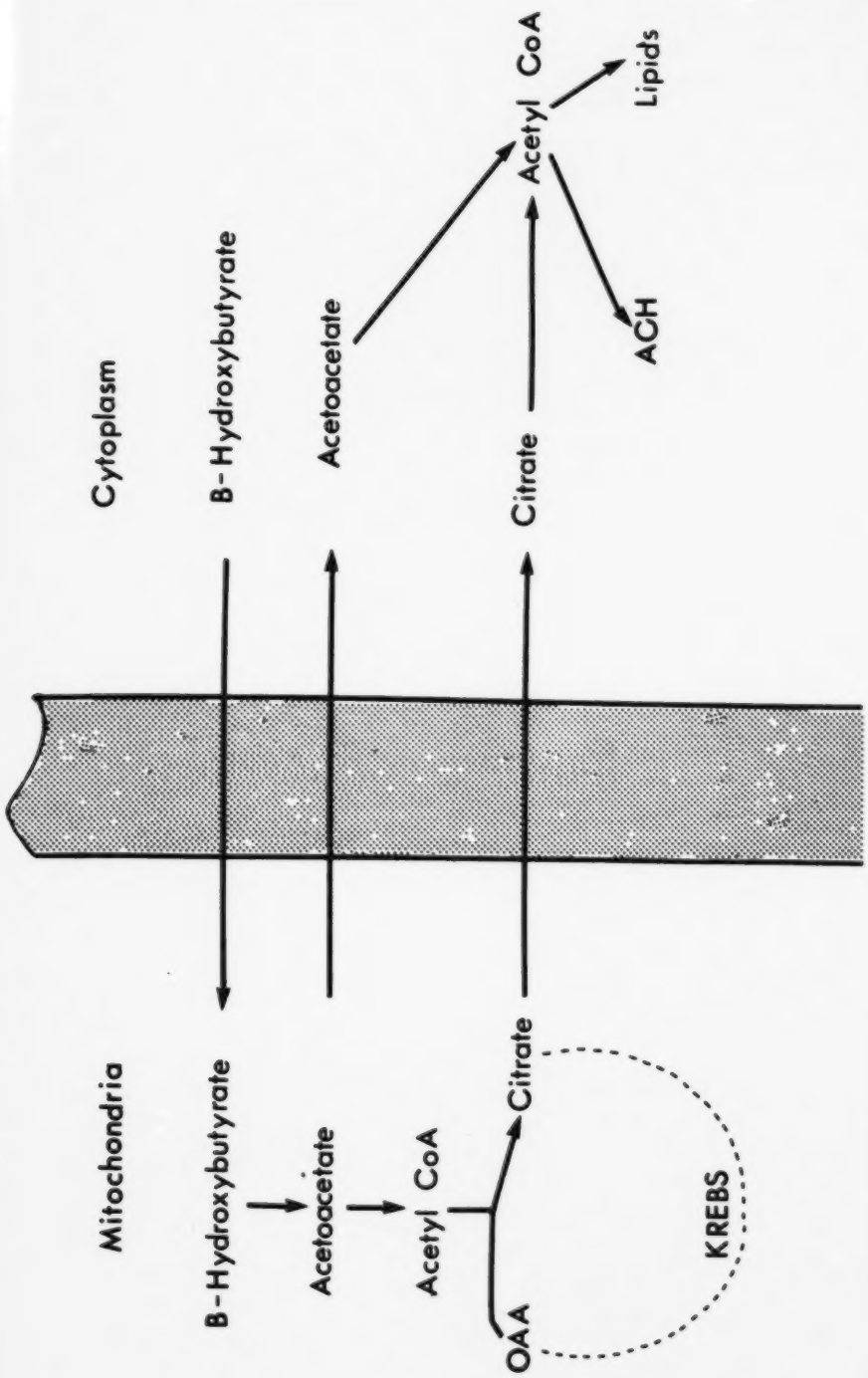

Figure 9. Mitochondrial and cytoplasmic acetyl CoA.

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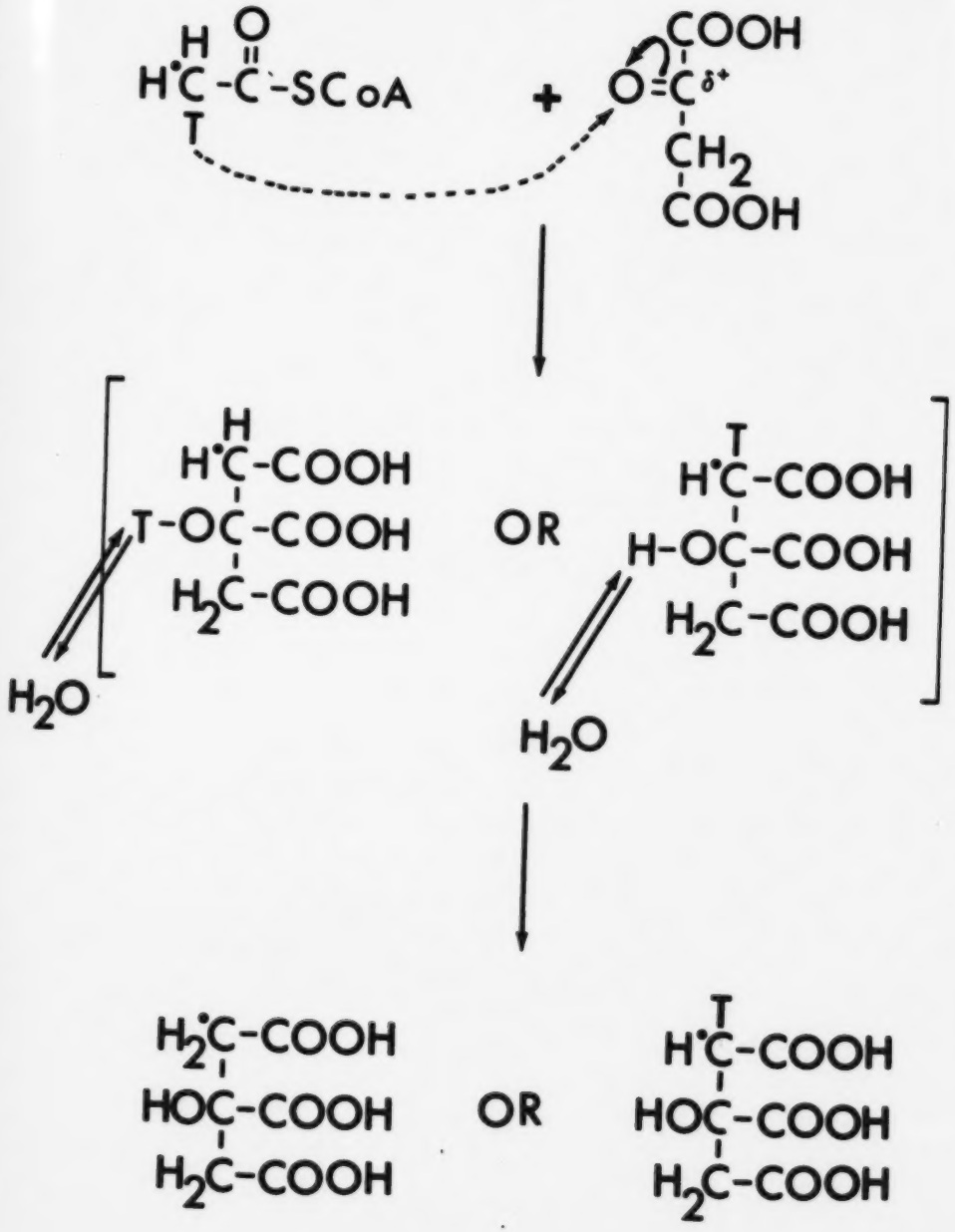

Figure 10. Loss of tritium in the citrate condensing reaction.

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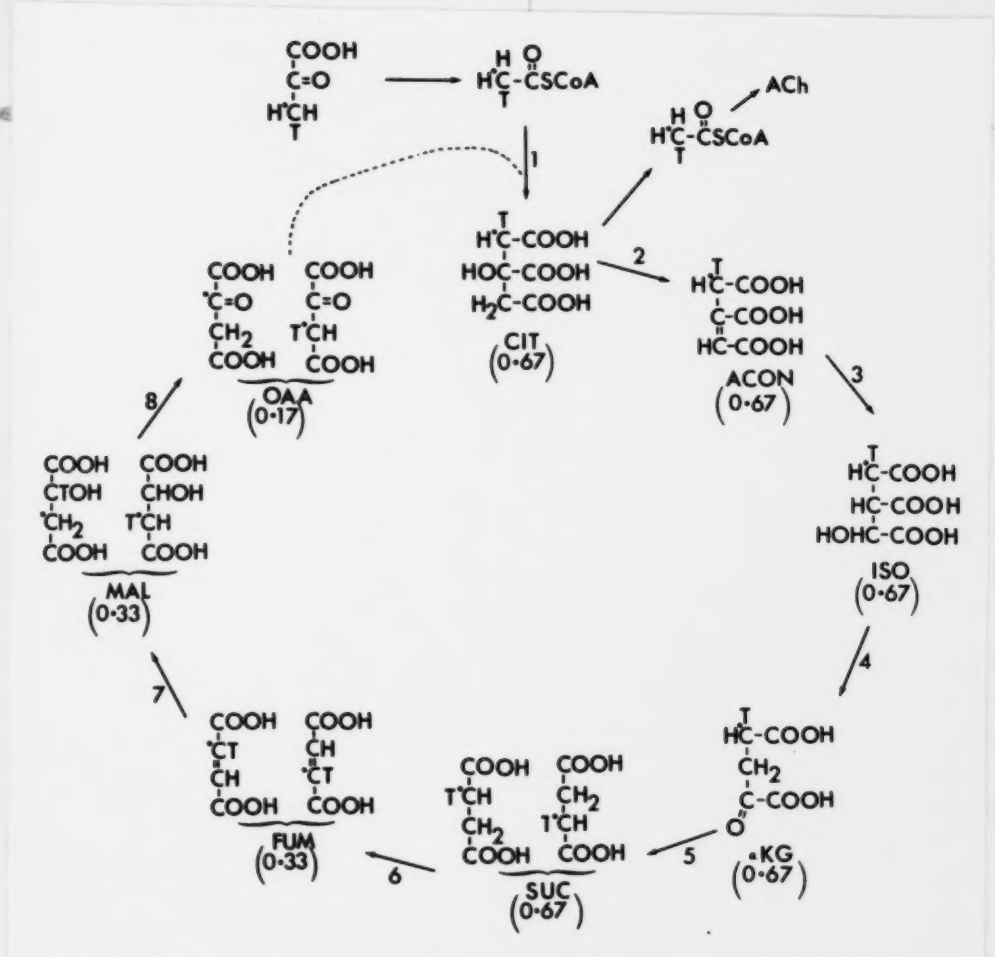

Figure 11. Changes in $^{3}H/^{14}C$ ratio with one turn of the Krebs cycle.

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TABLE 5. INFLUENCE OF POTASSIUM STIMULATION (105 mM) ON CONVERSION OF ^{3}H and ^{14}C of [6-^{3}H, 6-^{14}C] GLUCOSE TO ACETYLCHOLINE AND ITS PRECURSORS: CONTROL FOR COMPARISON OF Pb DATA.

	Radioactivity recovered in product		
Product	^{3}H	^{14}C	$^{3}H/^{14}C$ (n)
Lactate	22.2 ± 0.6	24.4 ± 0.9	0.91 ± 0.02* (4)
Citrate (total)	0.102 ± 0.007	0.276 ± 0.024	0.37 ± 0.01* (4)
Citrate (4-,5-)	0.069 ± 0.006	0.106 ± 0.007	0.65 ± 0.02* (3)
Malate	0.041 ± 0.003	0.143 ± 0.004	0.29 ± 0.02* (4)
Acetylcholine	0.015 ± 0.001	0.021 ± 0.001	0.71 ± 0.01† (7)

Experimental conditions are described under Methods and data treated as in Table 1.

*Ratio is not significantly different ($P > 0.05$) from unstimulated tissue values.

†$P < 0.05$ compared to unstimulated tissue.

TABLE 6. THE EFFECTS OF Pb^{++} (600 ppm) IN DRINKING WATER ON ACETYLCHOLINE SYNTHESIS [1] FROM [6-^{3}H, ^{14}C-] GLUCOSE.

Product	Radioactivity Recovered in Product		
	^{3}H	^{14}C	$^{3}H/^{14}C$ Ratio (n)
Lactate	15.1 ± 0.41*	15.7 ± 0.32*	0.96 ± 0.01
Citrate (total)	0.073 ± 0.001	0.205 ± 0.005	0.36 ± 0.01
Citrate (4-,5-)	0.031 ± 0.002	0.051 ± 0.003	0.61 ± 0.01
Malate	0.028 ± 0.002	0.106 ± 0.012	0.26 ± 0.01
Acetylcholine	0.009 ± 0.002	0.012 ± 0.0005	0.68 ± 0.01

Experimental conditions are described under Methods.

*When values are significantly greater than control ($p < 0.01 - 0.001 >$).

[1]See Table 1 for control conditions.

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TABLE 7. INFLUENCE OF POTASSIUM STIMULATION (105 mM) ON CONVERSION OF ^{3}H OF [6-^{3}H] GLUCOSE AND [3-^{14}C] β-HYDROXYBUTYRATE TO ACETYLCHOLINE AND ITS PRECURSORS.

	Radioactivity recovered in product		
Product	^{3}H	^{14}C	$^{3}H/^{14}C$ Ratio (n)
Lactate	23.6 ± 1.3	0.413 ± 0.02	57.1 ± 4.2 (5)
Citrate (total)	0.094 ± 0.006	0.642 ± 0.036	0.15 ± 0.01 (5)
Citrate (4-,5-)	0.049 ± 0.004	0.179 ± 0.014	0.27 ± 0.02 (5)
Malate	0.057 ± 0.003	0.394 ± 0.016	0.14 ± 0.01 (5)
Acetylcholine	0.014 ± 0.002	0.038 ± 0.003	0.37 ± 0.03 (5)

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TABLE 8. THE EFFECTS OF Pb^{++} (600 ppm) IN DRINKING WATER ON ACETYLCHOLINE SYNTHESIS FROM [^{3}H-6] GLUCOSE AND [^{14}-C] BETAHYDROXYBUTYRATE.

Product	Radioactivity recovered in product		
	^{3}H	^{14}C	$^{3}H/^{14}C$ Ratio (n)
Lactate	17.0	0.310	55.0 ± 2.7 (4)
Citrate (total)	0.009 ± .004	0.568 ± .049	0.17 ± 0.01
Citrate (4-,5-)	0.046 ± .003	0.157 ± .012	0.29 ± 0.01
Malate	0.033 ± .003	0.214 ± .012	0.15 ± 0.01
Acetylcholine	0.007 ± .0007	0.016 ± .001	0.44 ± 0.03

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sure affects synaptogenesis, an alcohol-phosphotungstate (EPT) method according to Agajanian and Bloom was employed and the two methods compared (Fig. 18 a,b - osmium; Fig. 19 a,b - EPTA). Complete descriptions of the perfusion, fixation and visualization procedures have been described (Progress Report, April 1, 1977 - March 31, 1978).

A series of 5 groups of 20 pictures and 1 group of 15 pictures resulting from EPTA procedure have been prepared and are being analyzed. These groups of photomicrographs cover the span from fetal through postnatal (day 15) for 200 ppm lead exposed and control animals.

There were significant changes in the appearance of both the transmission EM's and those treated with alcohol-EPTA as a function of development. Especially striking are changes occurring between day 16 through day 18 in the thoracolumbar sections of spinal cord. The use of 10 naive readers failed to detect any consistent differences between treated and control tissues up through day 15 of postnatal life. Two recent papers employing a similar comparison in cerebellar tissue in situ and in tissue culture found that not all synapses stained by the Osmic acid method were visible by the EPTA technique (Burry, 1977/78). This may explain in part the variability experienced with spinal cord.

MUSCARINIC RECEPTOR BINDING

In the developing spinal cord, acetylcholine may be important to the functional maturation of the synapse (Burt, J. Expt. Neurol. 169:107, 1968). A useful marker in this regard is the appearance with time of an increased population of acetylcholine receptors (AChR). There are two distinct AChR types in central and peripheral nervous tissue. One of these, the muscarinic receptor, is known to combine with the potent antagonist quiniclidine benzillate and its tritium labeled form QNB-3. Based on Burt's studies and those in this laboratory, it was of interest to extend earlier studies on acetylcholine synthesis and degradation in developing spinal cord to the ontogenetic development of the acetylcholine receptor.

Shown in Fig. 12 are the changes in receptor binding with fetal and postnatal development expressed as total drug bound per milligram protein. The control values (solid line) appear to display a steady increase from postnatal day 4 onward whereas there is a decline in cords from lead-treated animals. It also appears that there is a delay in appearance of binding in lead-treated animals on day 19 prenatal. Since the values are corrected for alterations in protein content, the differences may be greater expressed as drug bound per spinal cord. When corrections are made for nonspecific binding (Fig. 13) the differences between lead-treated (hatched lines) and controls (solid lines) become reduced. There is still, however, a lag of a day (days 19-20) in the rise in binding AChR-binding of QNB-3.

The level of muscarinic binding is much less in the fetal and neonatal spinal cord compared with adult values (X) of control animals. There appears to be much less nonspecific binding in spinal cord (total specific = nonspecific) than observed in brain tissue. Although muscarinic receptors predominate over nicotinic receptors in brain (85% vs 15%), little is known concerning the

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Figure 18a. Osmic acid electron micrographs - Control.

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Figure 18b. Osmic acid electron micrographs - Pb__ treated (200 ppm).

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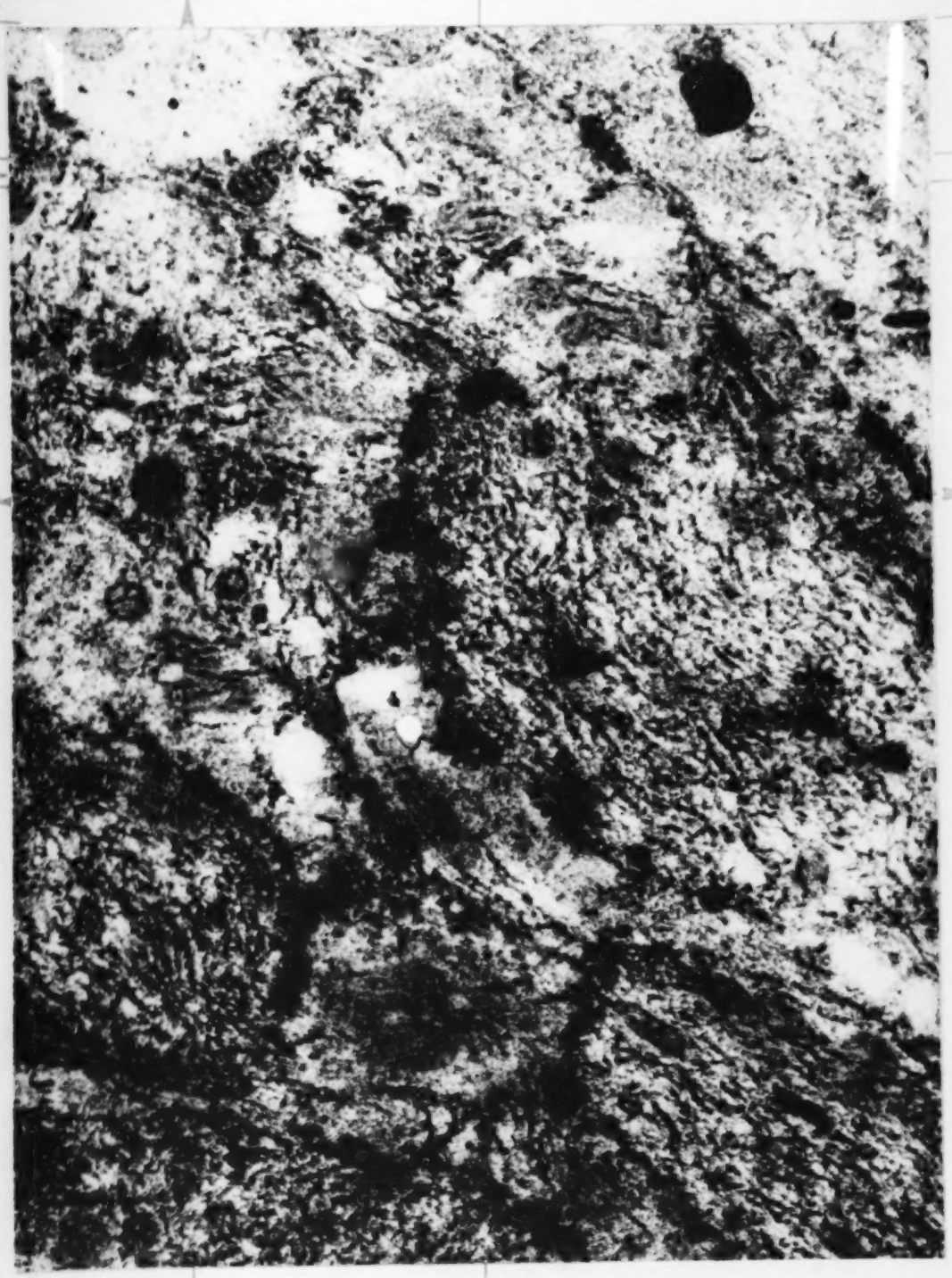

Figure 19a. EPTA electro micrographs - Control.

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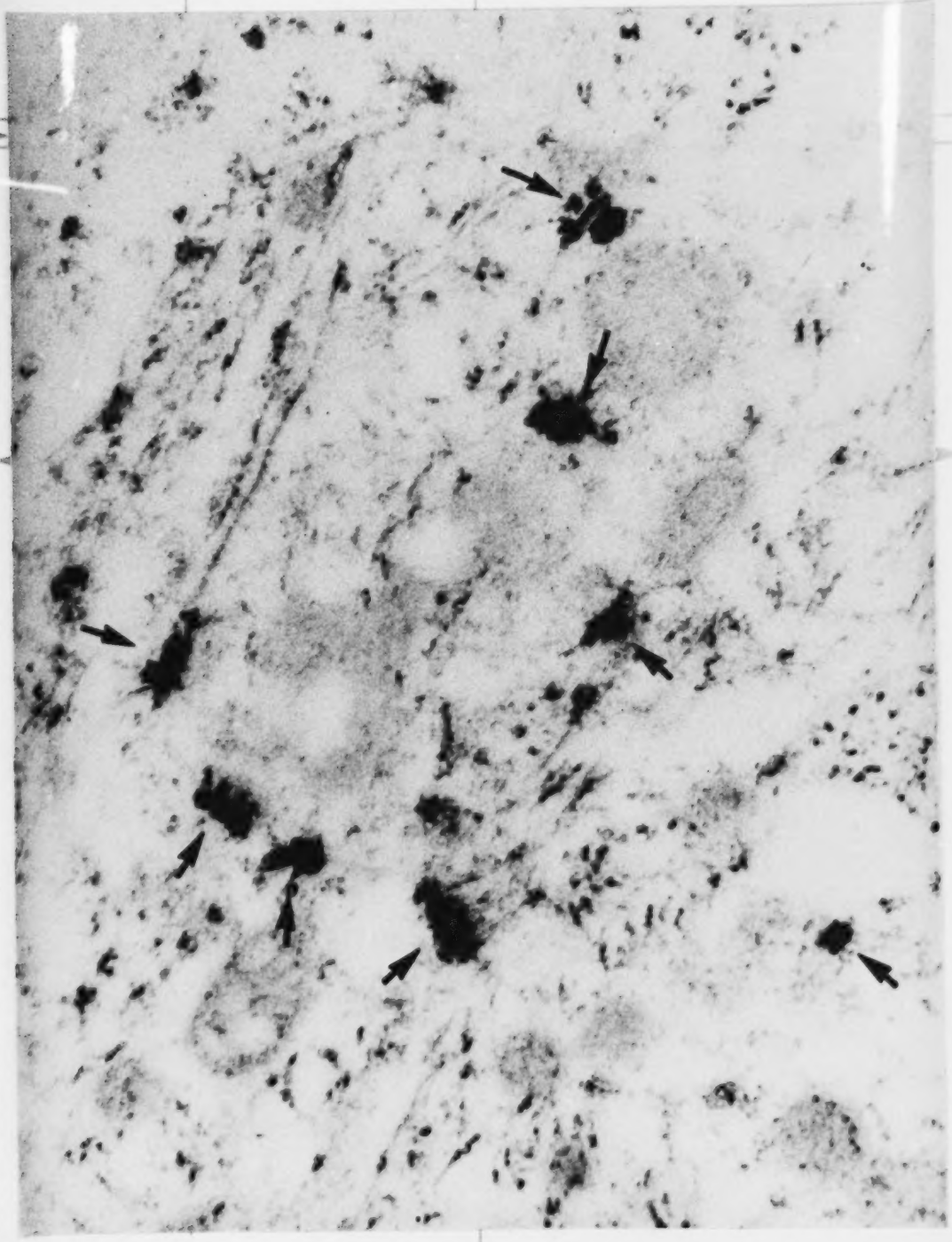

Figure 19b. EPTA electron micrographs - Pb^{++} treated (200 ppm).

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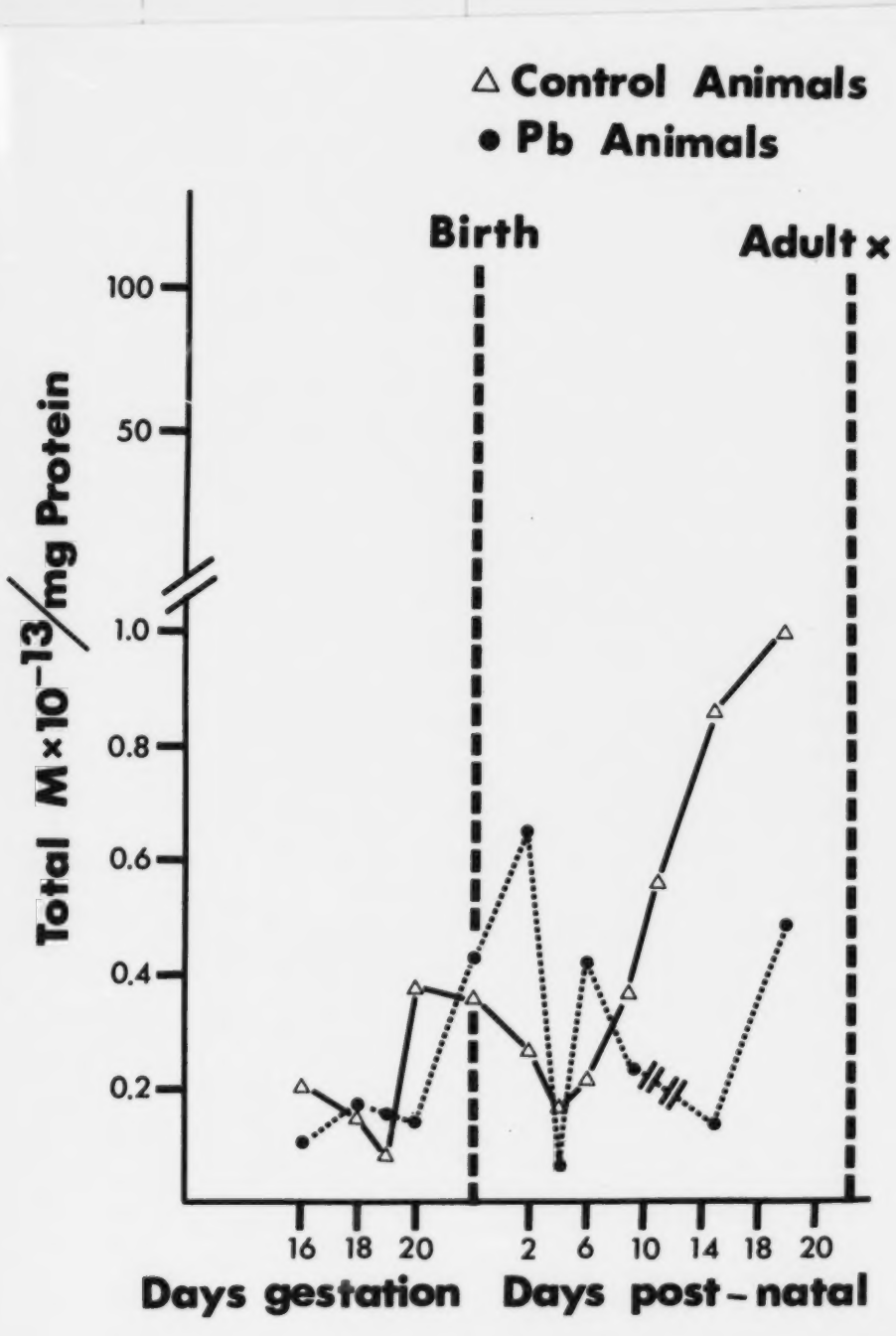

Figure 12. Total QNB binding in absence and presence of Pb treatment.

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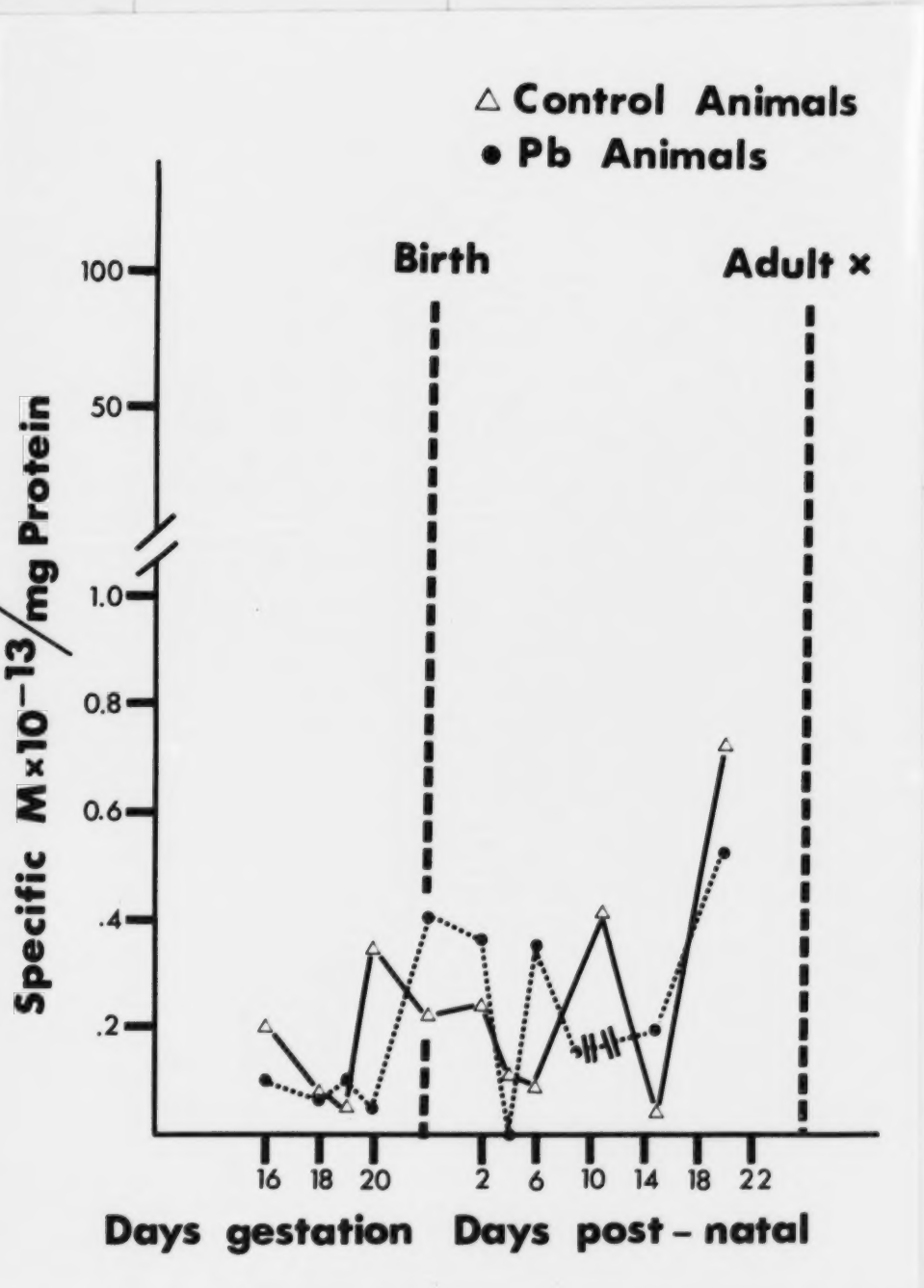

Figure 13. Specific QNB binding in absence and presence of Pb treatment.

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proportions in spinal cord. It would be interesting, therefore, to extend this study to include alpho-bungarotoxin binding as this is the most specific nicotinic receptor binding antagonist available. The changes in muscarinic binding roughly parallel the increase seen in cholineacetylase (Hruschak and O'Neill, 1975), and acetylcholinesterase by Burt (1968) as a function of development. The effects of lead on these parameters and on acetylcholine synthesis described in this study require further study asit relates to CNS development and its possible bearing on the "hyperactivity" syndrome reported by others.

Studies on the Effect of Lead on Carbonic Anhydrase "In Vitro" in Brain Subcellular Fractions and Kidney Cortex Microsomes

The studies on the effect of lead on the inhibition of carbonic anhydrase in "in vitro" have been finished. These studies show that particulate carbonic anhydrase of myelin and synaptasomes is highly sensitive to lead. Results are sumarized in Table 9.

Soluble carbonic anhydrase is practically not inhibited by lead at the concentrations studies. In the case of synaptosomes, however, inhibition can be shown at levels of 1 µg/ml, showing that the subcellular component is highly sensitive to lead.

Effect of Lead on "in Vivo" Brain Myelin, Nerve Endings and Kidney Microsomal Carbonic Anhydrase

The results of our initial studies on chronic lead intoxication of rats (600 ppm in drinking water) has been obtained. Sprague Dawley rats (180-200 g initial weight) were used. Diet of chow ad lib and acidified water (pH 5.0) containing 600 ppm lead acetate. A group of 36 rats were used for the experiment. They were divided into groups of 9 rats and every two weeks changes in CA were measured. For the analysis, groups of 3 rats corresponding to the period were sacrificed. The brains were pooled and subcellular fractionation was performed, isolating by sucrose density gradient, the myelin and nerve ending fractions. From the kidneys the microsomal fraction of the cortex was isolated by primary fractionation. Fractions were collected and kept in liquid nitrogen. The process was done for three consecutive days with the nine rats. The fractions were analyzed for CA, using pH electrode kinetic determination as described previously. Results are shown in Fig. 14. Initial conclusions are: (a) there is a decrease in brain carbonic anhydrase as a function of time; (b) the kinetics of this decrease is different for nerve endings and for the myelin, being the nerve endings more sensitive to low amounts of lead; (c) surprisingly, no significant changes are obtained in the kidney microsomal fraction.

Rats in the 8th week of intoxication were tested for seizure theshold using the fluorothyl methods. (In coll. with Dr. M.W. Adler). These animals showed a highly significant decrease in the threshold for convulsions(Table 10).

These results were surprising because we assumed that a decrease in brain carbonic anhydrase would lead to an increase in the threshold as shown pharmacologically with the sulfonamides.

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TABLE 9. "EFFECT OF LEAD ON CARBONIC ANHYDRASE "IN VITRO".

	CONTROL*	LEAD ACETATE (10 µg/mℓ)	% OF CONTROL	P
Myelin	5.11 ± 1.46	2.54 ± 0.75	49.71%	0.009
Nerve Endings	19.68 ± 3.79	2.37 ± 1.73	1.9%	0.00006
Kidney Cortex	15.05 ± 5.1	8.37 ± 3.11	56.0%	0.2

*Values are expressed as specific activity, units/mg p. One enzyme unit is a micromole of product formed per minute.

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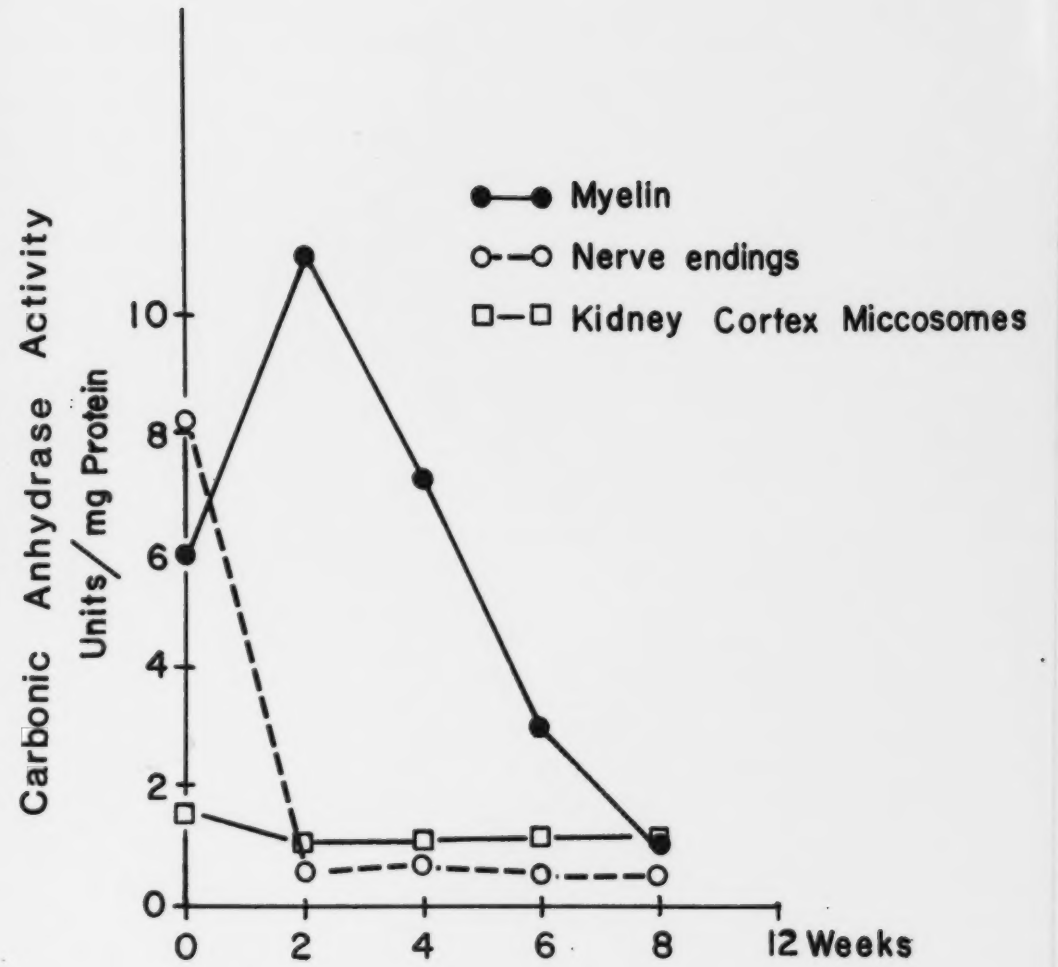

Figure 14. Effect of Pb^{++} concentration on various carbonic anhydrases.

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TABLE 10. THRESHOLD OF CONVULSION.

	PRECLONIC	CLONIC
Normal (sec)	250 ± 9	350 ± 9
Lead Intoxicated	211 ± 17	278 ± 28

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EFFECTS OF TRIBUTYL-TIN IN VITRO, ON LIVER SLICES

Tributyl-tin was studied with liver tissue rather than kidney cortex for the following reasons: no nephrotoxic effects of the compound have been reported; most work on the effects of this agent on mitochondrial metabolism have been carried out with liver organelles, so that much background work is available with this tissue; the CNS toxicity of trialkyl-tin compounds is accompanied by cerebral swelling and derangement of ion composition (Magee et al., 1957), and we have previously done much work to elucidate the volume controlling activity of cells, making use of liver as the model tissue. Accordingly, the effects of tributyl-tin were studied on water and ion exchanges, and energy metabolism, of rat liver slices.

Tributyl-tin has a number of effects on isolated mitochondria which result in a reduction of ATP formation by oxidative phosphorylation (Aldridge & Cremer, 1955; Aldridge & Street, 1971; Skilleter, 1975). Fig. 15 illustrates a titration of liver-slice energy metabolism with tributyl-tin, from which it can be seen that 1 mM reduced respiration and slice ATP contents by about 25%, while lower concentrations were without effect.

Many of the effects of tributyl-tin on mitochondria are dependent on the presence of Cl^-, the agent promoting a passage of this anion through the mitochondrial membranes in exchange for OH^- (Selwyn et al., 1970). Volume controlling activity in liver cells is partially due to an ouabain-resistant activity involving a net secretion of Na^+ and Cl^- accompanied by water (Macknight et al., 1974; Russo et al., 1977) and it was, therefore, of interest to examine the effect of tributyl-tin on this mechanism. Fig. 16 shows that, indeed, there was a marked inhibition of Cl^- extrusion from the slices which was evident even at a concentration of 1 µM tributyl-tin. The inhibition of Cl^- loss is tentatively interpreted as being due to an increased leakage of Cl^- into the cells by a passive mechanism facilitated by tributyl-tin, such as lead tending to negate the effects of the mechanism bringing about the net active extrusion of this ion. However, it can be seen that a marked increase of the effect was given a 1 mM tributyl-tin, at which concentration the inhibition of mitochondrial energy metabolism augmented the direct effect of tributyl-tin on Cl^- movements at the plasma membrane.

The decrease of net Cl^- extrusion was accompanied at all concentrations of the tin compound by distinct, but less marked, trends of inhibition of extrusion of Na^+ and water. However there was no effect on K^+ accumulation until the inhibitor concentration reached 1 mM - i.e., the concentration at which marked inhibition of O_2 consumption was seen. Thus, we conclude that the lower concentrations of tributyl-tin do not affect the coupled system for the transport of Na^+ and K^+, but only the mechanism bringing about the ouabain-resistant mechanism of volume control. Further experiments are now being conducted to study directly the effect of tributyl-tin on the ouabain-resistant movements of ions and water. Should these experiments confirm our conclusion, tributyl-tin may well prove a useful agent for the study of the ouabain-resistant system (for which no specific inhibitor has yet been detected), and furthermore, it will seem very likely that the neurotoxic effects of trialkyl-tin compounds are due to an inhibition of volume control, with consequent oedema.

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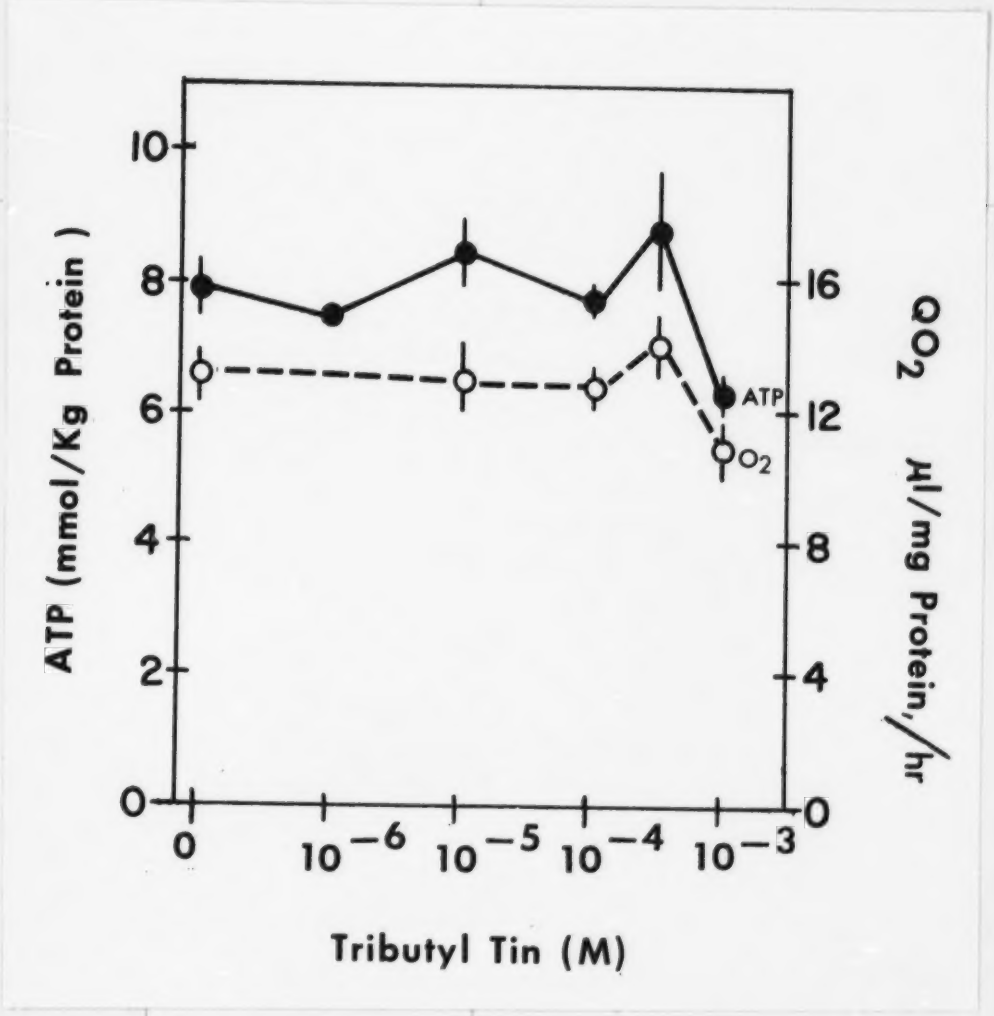

Figure 15. Influence of tributyl-tin concentration on QO_2 and ATP concentration.

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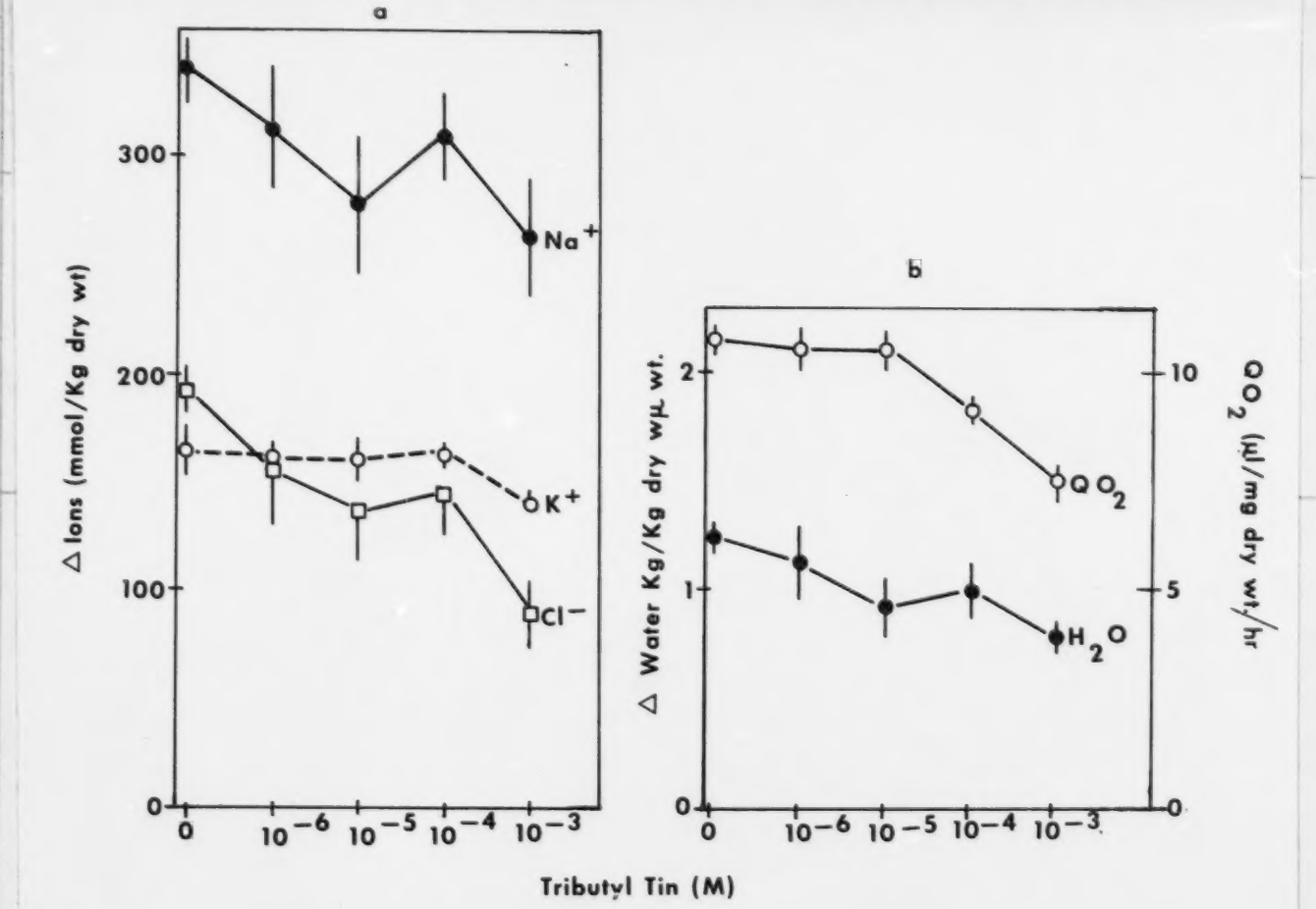

Figure 16a, b. Effects of tributyl-tin concentration; (a) on tissue concentrations of Na^+, K^+, and Cl^- ions; (b) on QO_2 and changes in tissue water content.

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